

General Bacteriology

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Introduction

Microbiology is the study of living organisms of microscopic size. This term was introduced by French chemist Louis Pasteur, who demonstrated that fermentation was caused by the growth of bacteria and yeasts. **Medical microbiology** is the study of microorganisms (bacteria, viruses, prions, fungi and parasites) capable of infecting and causing disease in humans, and the prevention, diagnosis and treatment of infectious diseases. It also deals with the response of the human host to microbial infection. Although the primary interest is in the diseases caused by the microorganisms, it must also be appreciated that microorganisms play a critical role in human survival.

The normal commensal population of microbes participates in the metabolism of food products, provides essential growth factors against infections with highly virulent microorganisms, and stimulates the immune response. In the absence of these organisms, life as we know would be impossible.

The term **microbe** was first used by Sedillot in 1878, but it has now been replaced by **microorganism**. Microbes were probably the first living things to appear on the earth, and the study of fossil remains indicates that microbial infections and epidemic diseases existed thousands of years ago.

Infectious diseases have been the bane of mankind for centuries and continue to cause high morbidity and sufferings worldwide. Disease and death have always attracted the attention of the human mind. The emergence of acquired immunodeficiency syndrome (AIDS) as a major modern day scourge with tremendous public health importance has brought into limelight even those diseases which were considered rare in the past.

The construction and use of the compound microscope (*micro*, small; and *skop*, to see) was an essential prerequisite to study the microbial forms. To **Antonie van Leeuwenhoek** (1632–1723) must be ascribed the credit of placing the science of microbiology on the firm basis of direct observation. This Dutch maker of lenses from Holland devised an apparatus and technique which enabled him to observe and describe various microbial forms with accuracy and care.

He observed, drew and measured a large number of minute living organisms including bacteria and protozoa, and

communicated them to Royal Society of London in 1683. The significance of these observations was not realized then and to Leeuwenhoek the world of '*little animalcules*' represented only a curiosity of nature. Their importance in medicine and in other areas of biology came to be recognized two centuries later. Antonie van Leeuwenhoek first accurately described different shapes of bacteria (coccal, bacillary and spiral) and pictured their arrangement in infected material in 1683.



Antonie van
Leeuwenhoek
(1632–1723)

ORIGIN OF MICROBIAL LIFE

In 1856, **Louis Pasteur** (1822–1895) was commissioned by an industrialist of Lille to investigate the problem which had arisen in manufacture of alcohol. The beet juice, from which alcohol was derived, was contaminated with a grey material which interfered with alcohol production. During the course of investigation, his attention was abruptly focused on the role of microorganisms in alcohol fermentation and spoilage. Undesirable forms of life could be destroyed at temperatures of 50–60°C in a short period of time. Subsequently, this modified process of heating came to be known as **pasteurization**. Pasteur established that different types of fermentations were due to the activity of different kinds of microbes.



Louis Pasteur
(1822–1895)

John Tyndall (1820–1893), an English physicist, was able to explain satisfactorily the need for prolonged heating to eliminate microbial life from infusions. He concluded, by exposing infusions to heat for varying times, that bacteria existed in two forms—a heat-stable form and a heat-sensitive

form. It required either prolonged or intermittent heating to destroy heat-stable forms. Intermittent heating, now called **tyndallization**, killed both forms since between periods of heat treatment, the heat-stable forms changed to heat-sensitive forms.

Ferdinand Cohn (1828–1898) described heat-stable forms as **spores**. Spores as well as vegetative forms were responsible for the appearance of microbial life in inadequately heated infusions.

Joseph Lister (1827–1912), an English surgeon and contemporary of Pasteur, was among the first to appreciate the ramifications of the emerging **germ theory** of disease. He attributed the frequent disastrous consequences following repair of compound fractures to invasions by airborne microorganisms. Lister introduced antiseptics in surgery. By spraying carbolic acid on surgical instruments, wounds and dressings, he reduced surgical mortality due to bacterial infection considerably. He established the guiding principle of antiseptic for good surgical practice upon which the present day specialists depend. For this work he is known as ‘**father of antiseptic surgery**’.

THE DEVELOPING SCIENCE OF MICROBIOLOGY

In the course of studies, Pasteur introduced the techniques of sterilization and developed steam sterilizer, hot air oven and autoclave. **Robert Koch** (1843–1910), a German physician, perfected the bacteriological techniques, staining procedures and methods of obtaining bacteria in pure culture using solid media during his studies on the culture and characters of anthrax bacillus.



Robert Koch
(1843–1910)

The causative agents of various diseases were reported rapidly by different investigators. Robert Koch discovered bacillus of tuberculosis (1882) and *Vibrio cholera* (1883); Hansen described the leprosy bacillus in 1874; Neisser discovered the gonococcus in the pus discharge from urethra in 1879; Alexander Ogston in 1880 described the staphylococci in abscesses and suppurative lesions; Eberth observed the typhoid bacillus in 1880; Klebs (1883) and Loeffler (1884) observed and described the diphtheria bacillus; Rosenbach (1886) demonstrated the tetanus bacillus with round terminal spore; Weichselbaum (1887) described and isolated the meningococcus from the cerebrospinal fluid of a patient; Bruce (1887) identified the causative agent of Malta fever in 1905 and Schaudinn and Hoffmann described the spirochaete of syphilis.

As the agents were being reported in such profusion, it became necessary to introduce criteria for proving the claims that a microorganism isolated from a disease was indeed causally related to it. **Henle** indicated such criteria but were enunciated by Koch which consisted of guidelines for the association of particular microorganisms with specific infectious diseases.

Koch observed thread-like organisms in the blood of animals that had died of anthrax, a disease that was serious threat to farmers killing their sheep and cattle herds periodically. He cultivated anthrax bacteria in pure culture in clear sterile vitreous humor of an ox's eye. He then injected pure cultures of the bacilli into mice and showed that the bacilli invariably caused anthrax. On autopsy, the blood was swarming with the thread-like bacteria and reisolated them in the vitreous humor. The cycle was now complete.

KOCH'S POSTULATES

A microorganism can be accepted as the causative agent of an infectious disease only if following postulates, known as Koch's postulates, are satisfied (Fig. 1.1).

1. The microorganism must be present in the lesions in every case of the infectious disease.
2. It should be possible to isolate the microorganism in pure culture from the lesions.
3. Inoculation of the pure culture, by a suitable route, into a suitable laboratory animal should produce a similar disease.
4. It should again be possible to re-isolate the microorganism in pure culture from the lesions produced in the experimental animals.

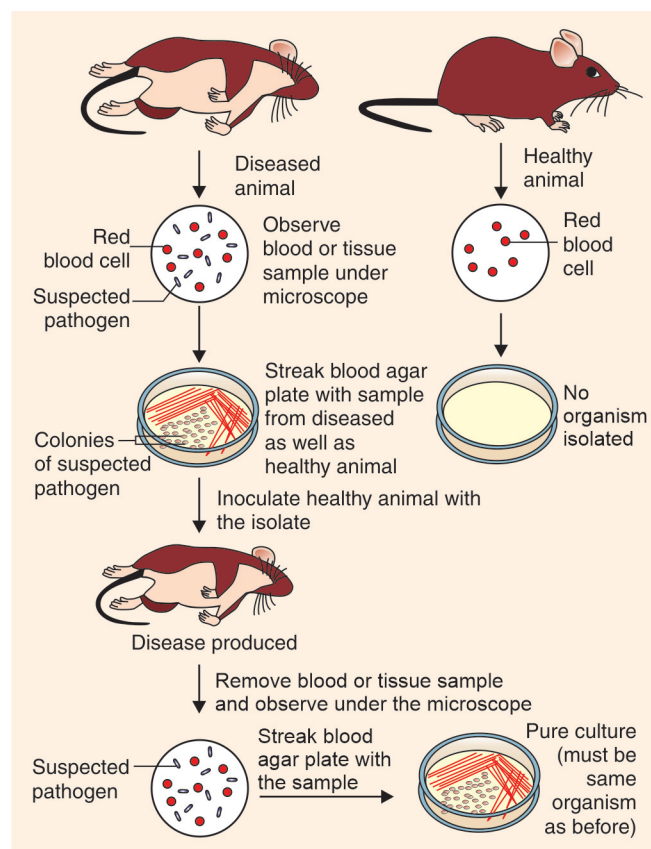


Fig. 1.1: Koch's postulates.

A **fifth criterion** introduced subsequently states that specific antibodies to the organism should be demonstrable

in the serum of the patient suffering from the disease. These postulates have proved extremely useful in confirming the authenticity of doubtful claims made regarding the causative agents of infectious diseases.

Exceptions to Koch's postulates

Koch's postulates have remained a mainstay of microbiology; however, many microorganisms that do not meet the criteria of Koch's postulates have been shown to cause disease. For example:

- *Treponema pallidum* and *Mycobacterium leprae*, causative agents of syphilis and leprosy, respectively, cannot be grown *in vitro*; however, there are animal models of infection with these agents.
- *Neisseria gonorrhoeae*, which causes gonorrhoea, there is no animal model even though the bacteria can readily be cultured *in vitro*.

THE BEGINNING OF VIROLOGY

For many years the term virus was used to describe any poison or microbial agent capable of causing an infection. In a large number of diseases such as smallpox, chickenpox, measles, influenza, poliomyelitis and the common cold, no bacterial cause could be established. Pasteur had suspected that rabies in dogs could be caused by a microbe too small to be seen under the microscope.

The first man to describe a filtered extract capable of causing disease in plants was Dmitri Iwanowski (1864–1920), a Russian scientist, who started his studies on diseases of tobacco while he was still a student. He reproduced mosaic disease in tobacco plant by applying juice from diseased plants to healthy leaves from which all bacteria had been removed by passage through fine filters (1892). In 1898, Martinus Beijerinck, unaware of Iwanowski's work, attributed the cause of tobacco-mosaic disease to *Contagium vivum fluidum*, a living liquid virus.

In 1898, Loeffler and Paul Frosch from Germany reported that the causative agent of foot-and-mouth disease in cattle would pass through a bacterial filter. Walter Reed (1902) in Cuba proved that the causative agent of yellow fever was not only a filterable virus but also transmitted through the bite of infected mosquitoes. The term 'filterable' was dropped in time and the tiny infectious agents were merely called **viruses**. Larger viruses could be seen under light microscope after appropriate staining but their detailed morphology could only be studied by electron microscope introduced by Ruska (1934). The technique of growing them on chick embryos developed by Goodpasture in 1930s and the application of cell culture in virology expanded the scope of virological techniques considerably.

Ellerman and Bang (1908) suggested the possibility that virus infection could lead to malignancy. Peyton Rous (1911) isolated a virus causing sarcoma in fowls. Several viruses have been blamed to cause natural and experimental tumours in birds and animals. Experimentally, viruses can cause malignant transformation of infected cells in tissue cultures.

The discovery of viral and cellular oncogenes have put forth the possible mechanisms of viral oncogenesis.

CONTRIBUTIONS OF VARIOUS SCIENTISTS IN THE FIELD OF MICROBIOLOGY

A large number of scientists has contributed in the field of microbiology. Principal contributions of some of them are given below.

Louis Pasteur

Louis Pasteur (1822–1895) was born in the village of Dole, France on December 27, 1822, the son of humble parents. His father was a tanner. He was originally trained as a chemist, but his studies on fermentation led him to take interest in microorganisms. His discoveries revolutionized medical practice, although he never studied medicine.

1. The term **microbiology**, as the study of living organisms of microscopic size, was coined by Pasteur.
2. He also coined the term **vaccine**.
3. He used various forms of nutrient fluid to grow microorganisms.
4. He showed that some organisms were not destroyed by boiling. For the sterilization of fluids he advocated heating to 120°C under pressure and for glassware the use of dry heat at 170°C. He showed that cotton plugs (a primitive air-filtration device) could prevent microbes from reaching otherwise air-exposed sterile broths.
5. In 1860–61, he disapproved the **theory of spontaneous generation**. In a series of classic experiments, Pasteur proved conclusively that all forms of life, even microbes, arose only from their alike and not *de novo*.
6. In 1860–64, he gave experimental evidence that fermentation and putrefaction are effects of microbial growth.
7. In 1863–65, he devised the process of destroying bacteria, known as **pasteurization**.
8. He introduced the techniques of sterilization and developed stream sterilizer, hot air oven and autoclave.
9. In 1877, Koch and Pasteur demonstrated that anthrax is caused by bacteria. Pasteur grew the organisms in sterilized yeast water and kept them in the laboratory for several months, transferring them frequently to new culture fluid, in which they multiplied readily, and showed that these cultures would always cause anthrax when inoculated into healthy animals.
10. In 1880, he prevented chicken cholera by injection of live attenuated culture. He found that pure cultures of the germ of this disease which had been kept in the laboratory for some time would not kill his animals as fresh cultures did, but would merely cause a passing illness from which the chickens recovered. Then he discovered that the animals that had recovered from a previous inoculation of weakened germs were immune, and did not succumb to the disease. Pasteur immediately perceived that it might be possible to make individuals

resistant by inoculating them with the weakened (and therefore harmless) germs of a particular disease.

11. In 1880, he first cultured staphylococci in liquid medium and produced abscesses by inoculating them into rabbits.
12. In 1881, he developed **live attenuated anthrax vaccine**.
13. In 1881, pneumococci were first noticed by Pasteur and Sternberg independently.
14. The crowning achievement of Pasteur was the successful application of the principle of vaccination to the prevention of rabies, or hydrophobia, in human beings and developed **Pasteur rabies vaccine** in 1885. He obtained fixed rabies virus by serial intracerebral passage in rabbits. The rabies vaccine was prepared by drying pieces of spinal cord from rabbits infected with fixed virus. Rabies vaccine prevented the development of this fatal disease if the inoculations are given soon after the bite of the rabid animal. He gave the first treatment for rabies in 1885 to a young boy bitten by a rabid dog.
15. In 1887, Pasteur and Joubert first described *Clostridium septicum* and called it *Vibrio septique*.

In 1888, in recognition of his incomparable achievements, the Pasteur Institute of Paris was built by public contribution during his lifetime for investigations of infectious diseases and preparation of vaccines. Acclaimed the world over for his epoch making discoveries, Pasteur died in Paris on September 28, 1895. His body lies in Pasteur Institute of Paris. Today the Pasteur Institute is a thriving research centre—an appropriate memorial to its founder.

Robert Koch

Contributions of Robert Koch, to microbiology, are variegated and enormous.

1. In 1876, Robert Koch reported the isolation of anthrax bacillus in pure culture, formation and germination of its spores and the proof of its infectiousness. This agent as the sole cause of anthrax was confirmed by Pasteur.
2. In 1877, he introduced the method of making smears of bacteria on glass slides, and of staining them with the aniline dyes.
3. In 1881, he described means of cultivating bacteria on solid media, thus making it possible to obtain pure cultures by transferring material from a single colony.
4. The **hanging-drop method** of studying bacteria as used today is a product of his genius.
5. In 1882, Koch startled the world by announcing his discovery of **tubercle bacillus** (*Mycobacterium tuberculosis*), the causative agent of tuberculosis. He described a special staining method for detection of this organism and grew it in pure cultures in the laboratory.
6. In 1883, he discovered the causative agents of cholera (*Vibrio cholerae*), Egyptian ophthalmia (pink eye) and Koch Weeks bacillus.
7. In 1884, Koch expounded the postulates or laws by which an organism may be proved to be the cause of a particular disease. These are known as **Koch's postulates**.

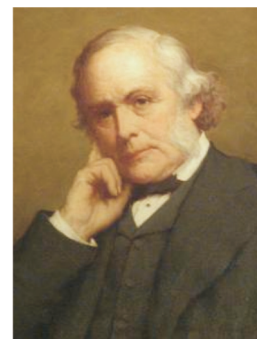
8. Koch continued his work on tuberculosis and in 1890–91 he showed how a normal guinea pig and an already infected guinea pig behaved differently to an infection with tubercle bacillus. This is known as **Koch's phenomenon** (see Chapter 28).

In 1905, he was awarded the **Nobel Prize in Medicine** for his work on tuberculosis. Together with Louis Pasteur, he laid the foundations of modern bacteriology.

Antonie van Leeuwenhoek

Antonie van Leeuwenhoek was expert in the grinding of simple magnifying lenses. He made these lenses of small bits of glass, polished them very carefully, and mounted each separately between two brass, copper, silver, or gold plates, to which he fastened an adjustable holder for the object to be examined. He constructed many of these 'microscopes' each containing a single lens ground by himself. The best of lenses magnified about 200 times. He observed, drew and measured a large number of living organisms including bacteria and protozoa in materials such as rain water, pond and well water, and saliva and the intestinal contents of healthy subjects and communicated them to the Royal Society of London in 1683. He was the first to accurately describe different shapes of bacteria (coccal, bacillary and spiral) and picture their arrangement in infected material.

Leeuwenhoek observed that very large numbers of bacteria appeared in watery infusions of animals or vegetable matter which were left to stand for a week or two at room temperature. He believed that these huge populations were the progeny of a few parental organisms, or seeds, that were originally present in the materials of the infusion or had entered it from the air. The significance of these observations was not realized then and to Leeuwenhoek the world of '*little animalcules*' represented only a curiosity of nature. Their importance in medicine and other areas of biology came to be recognized two centuries later.



Edward Jenner
(1749–1823)

Edward Jenner

Edward Jenner introduced the modern method of vaccination to prevent smallpox. He observed that milkmaids who contracted cowpox or vaccinia while milking were subsequently immune to smallpox. On May 14, 1796, he devised a brave experiment. He performed a vaccination against smallpox by transferring material from a cowpox pustule on the hand of a milkmaid, Sarah Nelmes, to the arm of a small boy named James Phipps, his gardener's son. Six weeks later the boy was inoculated with smallpox. He failed to develop the disease. *The terms vaccine and vaccination were first used by Pasteur out of deference to Jenner.*

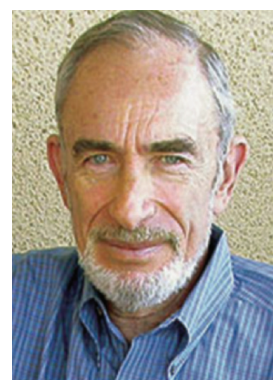
In 1967, the World Health Organization masterminded a final global plan to eradicate smallpox. Success was announced in 1980 with the declaration: Smallpox is dead.

Thanks to Jenner. Edward Jenner's discovery has now been developed into one of the most important parts of modern medicine—**immunology**.

Paul Ehrlich

1. In 1882, he reported the *acid-fastness* of tubercle bacillus.
2. From 1890 to 1900, he did important research in immunology. He soon found that the specific effect of immune serum could be demonstrated *in vivo* and *in vitro* and introduced methods of standardizing toxin and antitoxin. To him goes the credit of *minimum lethal dose*.
3. In 1898, he proposed *side chain theory of antibody production* (see Chapter 15).

4. In 1909, he introduced salvarsan, an arsenical compound, sometimes called the '*magic bullet*'. It was capable of destroying the spirochaete of syphilis with only moderate toxic effects. He continued his experimentation until 1912 when he announced the discovery of neosalvarsan. Thus he created a new branch of medicine known as *chemotherapy*.



Paul Ehrlich
(1854–1915)

KEY POINTS

- Microbiology is the biology of **microscopic organisms**, its subjects being microorganisms.
- Microorganism is an organism that **cannot be seen without the use of a microscope**.
- **Medical microbiology** deals with those **organisms which are responsible for infectious diseases of humans**.
- A microorganism is generally accepted as the causative agent of an infectious disease if it satisfies **Koch's postulates**.
- *Treponema pallidum*, *Mycobacterium leprae* and *Neisseria gonorrhoeae* do not fulfil all the criteria of Koch's postulates; the first two **cannot be grown in vitro** and for the third there is **no animal model**.

Important Questions

Write short notes on:

- (a) Koch's postulates.
- (b) Contributions of Louis Pasteur in microbiology.
- (c) Contributions of Robert Koch in microbiology.
- (d) Contributions of Antonie van Leeuwenhoek in microbiology.
- (e) Contributions of Edward Jenner in microbiology.
- (f) Contributions of Paul Ehrlich in microbiology.

Multiple Choice Questions

1. Which of the following organisms **does not** meet all the criteria of Koch's postulates?
 - (a) *Streptococcus pneumoniae*.
 - (b) *Treponema pallidum*.
 - (c) *Leptospira interrogans*.
 - (d) *Mycobacterium scrofulaceum*.
2. The construction and use of the compound microscope is attributed to:
 - (a) Antonie van Leeuwenhoek.
 - (b) Louis Pasteur.
 - (c) Robert Koch.
 - (d) Ferdinand Cohn.
3. Salvarsan was discovered by:
 - (a) Karl Landsteiner.
 - (b) Paul Ehrlich.
 - (c) Gerhardt Domagk.
 - (d) Howard Florey.
4. Bacillus of tuberculosis was discovered by:
 - (a) Hansen.
 - (b) Loeffler.
 - (c) Robert Koch.
 - (d) Bruce.
5. The term microbiology, as the study of living organisms of microscopic size, was coined by:
 - (a) Antonie van Leeuwenhoek.
 - (b) Robert Koch.
 - (c) Louis Pasteur.
 - (d) Edward Jenner.
6. The term vaccine was coined by:
 - (a) Edward Jenner.
 - (b) Kitasato.
 - (c) Ehrlich.
 - (d) Louis Pasteur.

7. Rabies vaccine was developed for the first time in 1885 by:
 - (a) Louis Pasteur.
 - (b) Semple.
 - (c) Edward Jenner.
 - (d) Paul Ehrlich.
8. Who discovered *Mycobacterium tuberculosis* and *Vibrio cholerae*?
 - (a) Loeffler.
 - (b) Welch.
 - (c) Pfeiffer.
 - (d) Robert Koch.
9. Who introduced the method of vaccination to prevent smallpox?
 - (a) Louis Pasteur.
 - (b) Edward Jenner.
 - (c) Paul Ehrlich.
 - (d) John Hunter.
10. Who of the following scientists proposed side chain theory of antibody production in 1898?
 - (a) Paul Ehrlich.
 - (b) Elie Metchnikoff.
 - (c) John Hunter.
 - (d) Edward Jenner.
11. Living organisms including bacteria and protozoa were first observed by:
 - (a) Louis Pasteur.
 - (b) Robert Koch.
 - (c) Antonie van Leeuwenhoek.
 - (d) Christian Gram.
12. Who is known as father of antiseptic surgery?
 - (a) Alexander Fleming.
 - (b) Joseph Lister.
 - (c) Charles Nicolle.
 - (d) Christian Gram.
13. Attenuated vaccine was first developed by:
 - (a) Louis Pasteur.
 - (b) Robert Koch.
 - (c) Landsteiner.
 - (d) Ehrlich.
14. Which of the following pioneers of microbiology is credited with the discovery of microorganisms using high-quality magnifying lenses (early microscopes)?
 - (a) Robert Koch.
 - (b) Louis Pasteur.
 - (c) Antonie van Leeuwenhoek.
 - (d) Hooke.
15. Which of the following is **not** a condition of Koch's postulates?
 - (a) Isolate the causative agent of a disease.
 - (b) Cultivate the microbe in the laboratory.
 - (c) Inoculate a test animal to observe the disease.
 - (d) Produce a vaccine.



Answers

- | | | | | | | | | | | | |
|-------|-------|-------|------|------|------|------|------|------|-------|-------|-------|
| 1. b | 2. a | 3. b | 4. c | 5. c | 6. d | 7. a | 8. d | 9. b | 10. a | 11. c | 12. b |
| 13. a | 14. c | 15. d | | | | | | | | | |

Morphology of Bacteria

Bacteria are free-living, microscopic, unicellular organisms capable of performing all the essential functions of life, e.g. growth, metabolism and reproduction. They possess both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) and lack chlorophyll. Bacteria have been placed in a kingdom separate from the animal and plant kingdoms, **Monera**.

Cells that have a well-defined nucleus are called **eukaryotes** (*eu*, true; and *karyon*, nucleus), whereas cells that lack a well-defined nucleus are called **prokaryotes** (*pro*, primitive; and *karyon*, nucleus). Bacteria are prokaryotes, while fungi, algae, protozoa, plants and animals are eukaryotes. In general, the interior organization of eukaryotic

cells is more complex than that of prokaryotic cells. The comparison of prokaryotes and eukaryotes is given in Table 2.1.

SIZE OF BACTERIA

Bacteria are very small in size. The unit of measurement of bacteria is called micrometre (μm). One μm is a millionth part of a metre or a thousandth part of a millimetre (mm). One nanometre (nm) is a thousandth part of a μm , and one Angstrom unit ($\text{\AA}$) is one-tenth of a nanometre. The diameter of the smallest body that can be resolved and seen clearly with naked eye is about 200 μm .

Table 2.1: Comparison of prokaryotes and eukaryotes

Characteristics	Prokaryotes	Eukaryotes
Major groups	Bacteria	Algae, fungi, protozoa, plants and animals
Genetic material		
Location	Free in the cytoplasm attached to a structure called mesosome located in the cell membrane	Contained within a membrane-bound nucleus inside the cell
Form	A single circular piece of DNA	Multiple chromosomes, which are surrounded by basic proteins called histones
Nucleolus	Absent	Present
Replication	By binary fission	By mitosis and meiosis
Extrachromosomal DNA	Plasmids—small circular pieces of DNA containing accessory information, present in the cytoplasm	In mitochondria
Protein production site	No endoplasmic reticulum; ribosomes—free in the cytoplasm or attached to the cell membrane	- Rough endoplasmic reticulum, a membrane covered with ribosomes, where protein is made - Smooth endoplasmic reticulum or Golgi complex, where secreted proteins are packaged and transported to the cell surface
Ribosomes	70S in size, consisting of 50S and 30S subunits	80S in size, consisting of 60S and 40S subunits
Energy production site	Electron transport chain located in the cell membrane; no mitochondria present	Within membrane-bound mitochondria
Intracellular organelles (lysosomes)	Absent	Contain hydrolytic enzymes
Cytoplasmic membrane	With the exception of <i>Mycoplasma</i> , bacterial cytoplasmic membrane lacks sterols	Does contain sterols
Cell wall	Present; is a complex structure containing peptidoglycans, protein, and lipids	Usually absent except in fungi, which contain chitin in the cell wall

Medically important bacteria, generally, measure 0.2–1.5 μm in diameter and 3–5 μm in length. Therefore, to visualize most bacteria one must use the higher powers of magnification of a good light microscope and enlarge them about 1000 times. To visualize their surfaces distinctly, it is usually necessary to stain them. Electron microscopy is essential for clear visualization of internal structures of the bacteria.

Microscopy

The study of the morphology of bacteria requires the use of microscopes. Following types of microscopes are used for examination of bacteria.

Light microscope

Bacteria may be examined under light microscope, either in the living state or after fixation and staining. The arrangement, motility and approximate size of the organisms can be observed by the examination of wet films or 'hanging drops'. But, due to lack of contrast, details cannot be appreciated.

Phase-contrast microscope

The phase-contrast microscope takes advantage of the fact that light waves passing through transparent objects such as cells, emerge in different phases depending on the properties of the materials through which they pass. A special optical system converts difference in phase into difference in intensity, so that some structures appear darker than others. It can be used to reveal some details of the internal structures in living cells.

Dark-ground (dark-field) microscope

This microscope renders visible delicate organisms such as *Treponema pallidum*, a spirochaete which is less than 0.2 μm in diameter and therefore cannot be observed with direct light. Dark-field microscopy is frequently performed on the same microscope on which bright field microscopy is performed. By means of a special condenser with a circular central stop, the specimen is illuminated by oblique light only. The rays do not enter the tube of the microscope, and in consequence, do not reach the eye of the observer unless they are scattered by objects (e.g. bacteria) of different refractive index from the medium in which they are suspended. As a result, the organisms appear brightly illuminated against a dark background.

The advantage of this method is that the resolving power of the dark-field microscopy is significantly improved compared with that of bright-field microscopy. The disadvantage of this method is that the light passes around rather than through the organisms, making it difficult to study their internal structure.

Fluorescence microscope

When ultraviolet or short-wavelength or invisible light falls on a fluorescent substance, the wavelength of the invisible light increases, so that it becomes luminous and is said to fluoresce. If tissues, cells or bacteria are stained with a fluorescent dye and are examined under the micro-

scope with ultraviolet light instead of ordinary visible light, they become luminous and are seen as bright objects against a dark background. Moreover, fluorescent dyes have a selective action for various microorganisms and cells and for their constituents which thus become readily recognized and identified. The fluorochrome dyes auramine and rhodamine can be used to demonstrate acid-fast bacilli. Viewed by fluorescence microscopy, the bacterial cells appear yellow against a dark background when potassium permanganate is used as a counterstain. For immunofluorescence see Chapter 13.

Electron microscope

The greatly increased resolving power of the electron microscope (EM) has enabled scientists to observe the detailed structures of prokaryotic and eukaryotic cells. The superior resolution of the EM is due to the fact that electrons have a much shorter wavelength than the photons of white light. In EM, a beam of electrons is employed instead of the beam of light used in the optical microscope. The electron beam is focused by circular electromagnets, which are analogous to the lenses in the light microscope. The object which is held in the path of the beam scatters the electrons and produces an image which is focused on a fluorescent viewing screen.

The wavelength of electrons used in an EM is 0.005 nm, as compared to 500 nm with visible light, i.e. about 100,000 times shorter than that of ordinary light. Theoretically, if conditions were identical in the optical and electron microscopes, the resolving power of the EM should be 100,000 times (resolution down to 0.0025 nm). However, the numerical aperture of an EM lens is very small (the diameter of the aperture is only a few micrometers) and does not approach the width of that of an optical microscope objective. In practice, the best resolution that can be obtained is 0.3–0.5 nm, a hundred times better than that of the light microscope.

An important technique in electron microscopy is the use of 'shadowing'. This involves depositing a thin layer of heavy metal (such as platinum) on the specimen by placing it in the path of a beam of metal ions in a vacuum. The beam is directed at a low angle to the specimen, so that it acquires a 'shadow' in the form of an uncoated area on the other side. When an electron beam is then passed through the coated preparation in the EM and a positive print is made from the 'negative' image, a three-dimensional effect is achieved.

Another technique in electron microscopy includes the use of ultrathin sections of embedded material—a method of freeze-drying specimen, which prevents the distortion caused by conventional drying procedures; and the use of negative staining with an electron-dense material such as phosphotungstic acid or uranyl salts. This technique enables the study of cellular ultrastructure as it appears in the living state.

SHAPE OF BACTERIA

Bacteria exist in different shapes as under (Fig. 2.1):

1. **Cocci** (from *kokkos* meaning berry) are round or oval cells.

2. **Bacilli** (from *baculus* meaning rod) are rod or stick-shaped. In some of the bacilli the length of the cells may be equal to width. Such bacillary forms are known as **coccobacilli**. The latter have to be carefully differentiated from cocci.
3. **Vibrios** are curved or comma-shaped rods.
4. **Spirilla** are non-flexuous spiral forms with one to three fixed curves in their rigid bodies.
5. **Spirochaetes** (from *spira* meaning coil and *chaite* meaning hair) are slender and flexuous spiral forms.
6. **Mycoplasmas** are cell wall deficient organisms. Therefore, they do not possess stable morphology. They occur as round or oval bodies or as interlacing filaments.

GROUP PATTERNS

The most frequent method of reproduction among bacteria is asexual binary fission, that is each cell splits in half, forming two new cells. As they increase in number they form distinct groups. Cocci that split along one plane only tend to arrange themselves in pairs (**diplococci**) or in chains (**streptococci**). When the division occurs alternatively in each of two planes, groups of four (**tetrads**) or eight (**octads**) are formed. Haphazard splitting in several planes results in the formation of clusters of cocci (Fig. 2.1).

Bacilli split only across their short axes, therefore, the patterns formed by them are limited. They may appear as end-to-end pairs (**diplobacilli**), or chains (**streptobacilli**) (Fig. 2.1). In some instances, there occurs incomplete separation of the daughter cells after binary fission. The bacilli remain attached to each other at various angles, resembling the letters V or L. This is called **Chinese letter arrangement** and is characteristic of *Corynebacterium diphtheriae*.

ANATOMY OF A BACTERIAL CELL

The principal structure of a bacterial cell is shown in Fig. 2.2. The interior of the cell, the protoplast, is differentiated into cytoplasm and nuclear material. Cytoplasm is bounded by a thin, elastic and semipermeable cytoplasmic membrane. Outside this lies cell wall, which gives the bacterium its shape

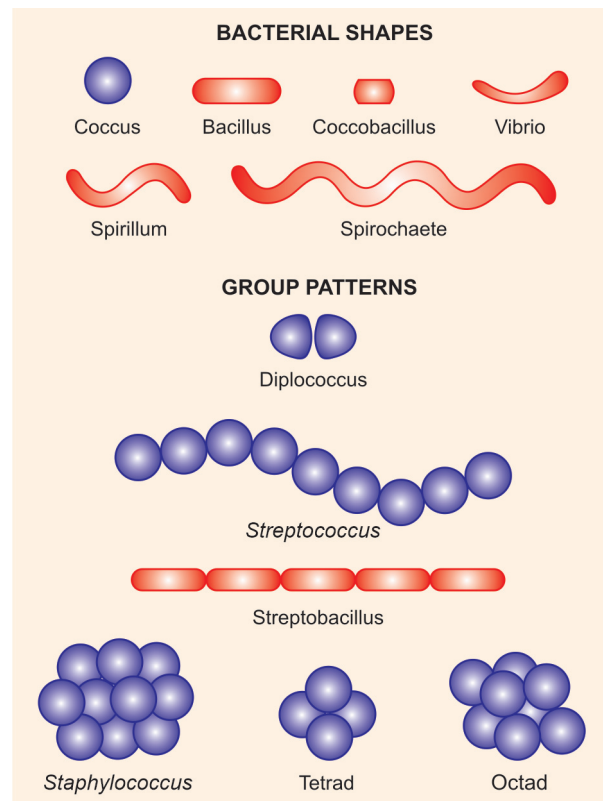


Fig. 2.1: Shapes and group patterns of bacteria.

and rigidity. Cell wall, in many bacteria, is enclosed by a protective gelatinous covering layer called capsule. Many bacteria also possess flagella which are the organelles of motility and some species have fimbriae (pili) too.

Bacterial cell wall

- It is a complex rigid structure which gives bacteria their definite shape.
- It is permeable to passage of liquid nutrient material into the cell, and to outward passage of substances produced within the cell.
- It is about 10–20 nm in thickness and constitutes 20–30% of dry weight of the cell.

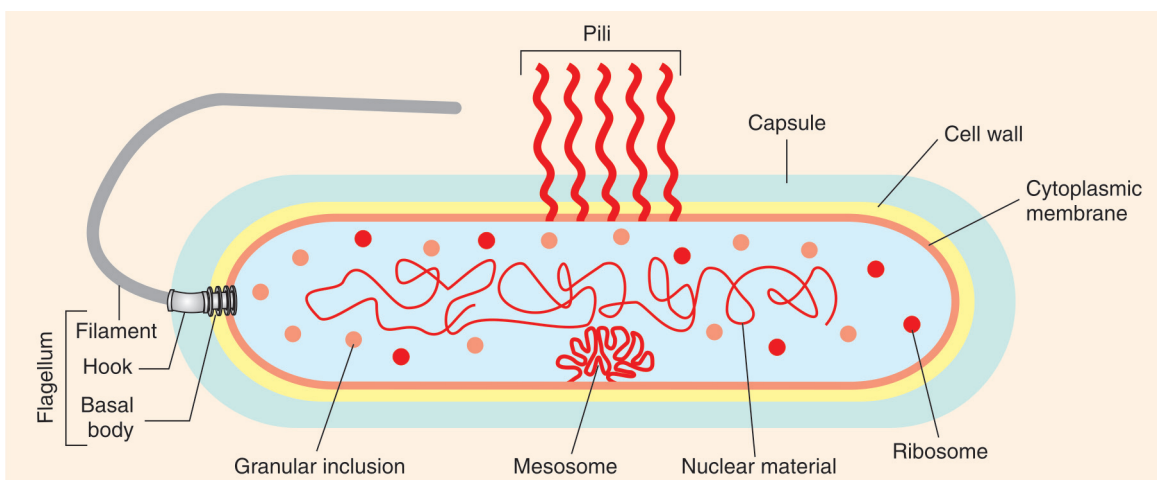


Fig. 2.2: Anatomy of a bacterial cell.

- The cell walls of Gram-positive bacteria are generally thicker than those of Gram-negative bacteria.
- The strength of the bacterial cell wall is due to the presence in it of a substance referred to as **peptidoglycan**, mucopeptide or murein.

Peptidoglycan consists of three parts—a backbone, composed of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid; a set of identical tetrapeptide side chains attached to *N*-acetylmuramic acid; and a set of identical pentapeptide cross-bridges (Fig. 2.3). In all bacterial species the backbone is the same, however, tetrapeptide side chains and pentapeptide cross-bridges vary from species to species.

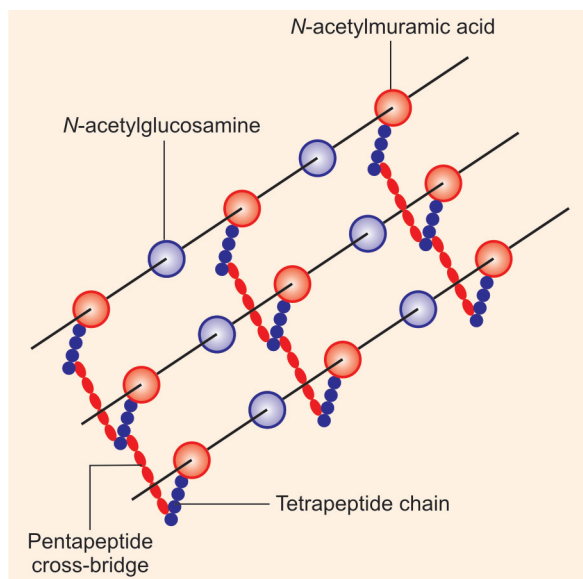


Fig. 2.3: Chemical structure of peptidoglycan.

Gram-positive bacterial cell wall

- The Gram-positive bacterial cell wall (Fig. 2.4) is 16–80 nm thick and is composed mostly of several layers of **peptidoglycan**.
- It constitutes **50–90%** of the dry weight of the wall.
- In addition to peptidoglycan, Gram-positive cell wall also contains teichoic acids and polysaccharides. There are two types of teichoic acids—cell wall teichoic acid, covalently linked to peptidoglycan; and membrane **lipoteichoic acid**, covalently linked to cytoplasmic membrane.

Gram-negative bacterial cell wall

- The cell wall of Gram-negative bacteria (Fig. 2.5) is thinner (2 nm) than that of Gram-positive bacteria but is structurally more complex. It consists of peptidoglycan, lipoprotein, outer membrane, and lipopolysaccharide.
- Peptidoglycan layer is a single-unit thick. It constitutes 5–10% of the dry weight of the wall of Gram-negative bacteria.
- Outside the thin peptidoglycan layer, is the outer membrane. In this membrane are embedded various large molecules like outer membrane protein and porin protein. Large antibiotic molecules penetrate outer membrane relatively slowly, which accounts for the relatively high

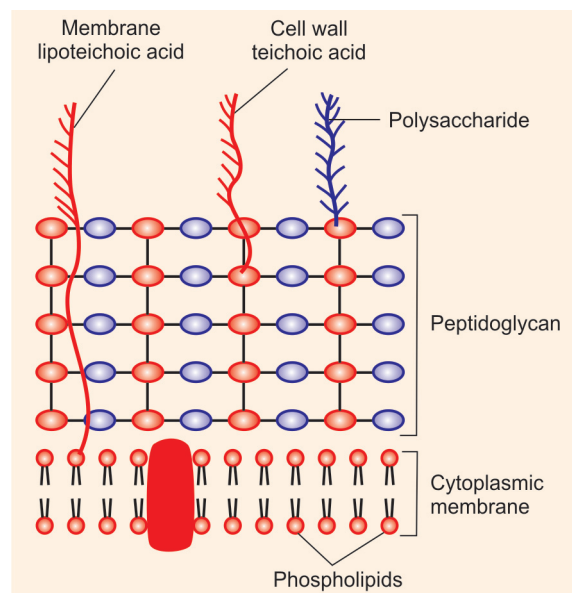


Fig. 2.4: Gram-positive cell wall.

antibiotic resistance of Gram-negative bacteria. Outer membrane is anchored to the peptidoglycan layer by strongly lipophilic lipoprotein.

- A structural component that is unique to the Gram-negative outer membrane is **lipopolysaccharide (LPS)**. It consists of a complex lipid, called lipid A, to which is attached a core polysaccharide. From core polysaccharide extends outward O polysaccharide. It has several peculiar sugars and varies in composition between bacterial strains, conferring species-specific antigen specificity. It represents a major surface antigen of the bacterial cell. It is known as **O antigen**. Bacteria carrying LPS containing O antigen form smooth colonies in bacteriological media in contrast to those lacking O antigen, which form rough colonies. LPS is firmly bound to the cell surface and is released only when the cells are lysed. It is, therefore, known as **endotoxin** and is extremely toxic to animals. All the toxicity of the endotoxin is due to lipid A.
- The space between the inner membrane (cytoplasmic membrane) and outer membrane is known as **periplasmic space**. It contains the peptidoglycan layer and a gel-like solution of proteins.

The differences between cell walls of Gram-positive and Gram-negative bacteria are given in Table 2.2.

Acid-fast cell wall

Certain genera (*Mycobacterium* and *Nocardia*) have a Gram-positive cell wall structure but, in addition, contain a waxy layer of glycolipids and fatty acids (**mycolic acid**) bound to the exterior of the cell wall. This makes *Mycobacterium* species difficult to stain with the Gram stain. The mycobacteria and nocardiae can be stained with an acid-fast stain, in which the bacteria are stained with carbol fuchsin, followed by treatment with sulphuric acid or acid alcohol as decolourizer. Other bacteria are decolourized, whereas mycobacteria and nocardiae retain the stain. They are, therefore, known as **acid-fast bacteria**.

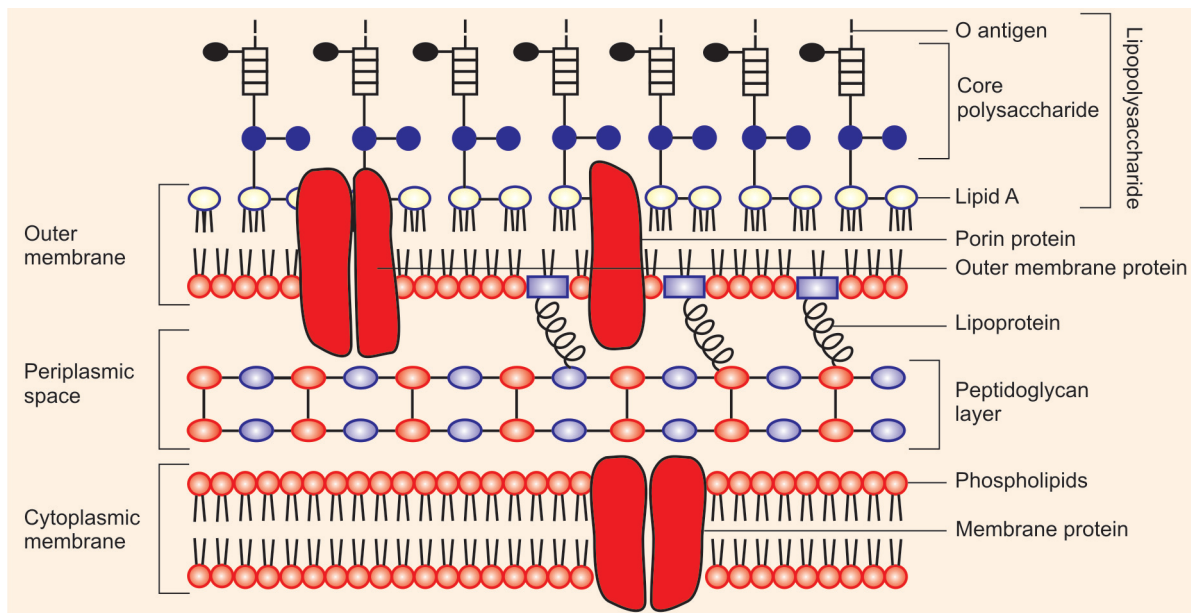


Fig. 2.5: Gram-negative cell wall.

Table 2.2: Differences between cell walls of Gram-positive and Gram-negative bacteria

Character	Gram-positive cell wall	Gram-negative cell wall
1. Thickness	Thicker	Thinner
2. Peptidoglycan	Thick layer (16–80 nm)	Thin layer (2 nm)
3. Lipid content	2–5%	15–20%
4. Teichoic acid	Absent	Present
5. Porin proteins	Absent	Present
6. Variety of amino acids	Few	Several
7. Periplasmic space	Absent	Present
8. Endotoxic activity	Absent	Present

Demonstration of cell wall

The cell wall cannot be seen by light microscopy and does not stain with simple dyes. It can be demonstrated by:

- Plasmolysis
- Microdissection
- Exposure to specific antibody
- Mechanical rupture of the cell
- Differential staining procedure
- Electron microscopy.

Protoplasts and spheroplasts

The cell wall may be removed by treating the bacteria with lysozyme, which is normally present in animal secretions (tears, saliva, nasal secretions) and in egg white. It acts by hydrolyzing linkages of the peptidoglycan backbone.

Protoplast

When a Gram-positive bacterium is treated with lysozyme in a hypotonic solution it lyses. If the osmotic strength of the solution is raised to balance the internal osmotic pressure of

the cell, a free protoplast is liberated consisting of cytoplasmic membrane and its contents.

Spheroplast

When Gram-negative bacteria are treated with lysozyme, the outer membrane of the cell wall prevents access of lysozyme unless disrupted by an agent such as ethylenediamine-tetraacetic acid (EDTA). Lysozyme-EDTA treated bacteria result in the formation of spheroplasts which still possess remnants of the complex Gram-negative cell wall.

Removal of the bacterial cell wall may also be accomplished by growing the organisms in the presence of a substance such as penicillin, bacitracin or cycloserine that blocks peptidoglycan biosynthesis. A similar result may be obtained by growing the organisms on a medium lacking nutrients like diaminopimelic acid, lysine or hexosamine which are essential for cell wall synthesis.

If maintained on osmotically protective medium, protoplasts metabolize and grow in size but they do not multiply. Spheroplasts, on the other hand, when kept on osmotically protective agar medium containing cell wall inhibitor such as penicillin, may multiply by fission or budding and reproduce through many serial subcultures.

Because spheroplasts retain a residual cell wall, therefore, they are osmotically less sensitive than protoplasts and are often capable of growing on an ordinary agar medium. Spheroplasts are capable of reverting to parent bacterial form when cell wall inhibitor is removed from the culture medium. Protoplasts perhaps cannot do so. Because of their resemblance with L-forms of bacteria, spheroplasts may be called **unstable L-forms**.

Cytoplasmic membrane

Bacterial cytoplasmic membrane, also called cell membrane, limits the bacterial protoplast externally. It is thin (5–10 nm), elastic and consists of a phospholipid bilayer in which various

constituent proteins are embedded (Figs 2.4 and 2.5). With the exception of *Mycoplasma*, bacterial cytoplasmic membrane lacks sterols. **It acts as a semipermeable membrane controlling the inflow and outflow of metabolites to and from the protoplasm.** It permits the passive diffusion of water and other small molecular substances inward and outward, but it actively effects the selective transport of specific nutrients into the cell and that of waste products out of it. This is mediated through specific enzymes (permeases) present in the cytoplasmic membrane.

Cytoplasm

Cytoplasm of the bacterial cell is a viscous watery solution of soft gel, containing a variety of organic and inorganic solutes. It contains all biosynthetic components required by the bacterium for the growth and cell division, together with genetic material. The cytoplasm of bacteria differs from that of higher eukaryotic organisms in not containing endoplasmic reticulum, Golgi apparatus, mitochondria, lysosomes and in not showing signs of internal mobility, e.g. cytoplasmic streaming, the formation, migration and disappearance of vacuoles and amoeboid movement. Cytoplasm contains ribosomes, mesosomes and intracytoplasmic inclusion bodies (Fig. 2.2).

Ribosomes

Ribosomes are composed of ribosomal RNA (rRNA) and ribosomal proteins and are designated by their sedimentation coefficient (S or Svedberg unit). They are slightly smaller than those of eukaryotic cells. They measure 10–20 nm in diameter and have a sedimentation coefficient of 70S. Each 70S particle is composed of a 30S and a 50S subparticle. Each cell contains thousands of ribosomes strung together on strands of messenger RNA (mRNA) to form polysomes and it is at this site that code of mRNA is translated into peptide sequences. There are certain considerable differences between bacterial and host cell ribosomes. This allows us to use antibacterial agents such as streptomycin which interfere with bacterial metabolism at the ribosomal level without unduly upsetting human ribosomal function.

Mesosomes

These are convoluted or multilaminated membranous bodies which develop by invagination of cytoplasmic membrane into the cytoplasm (Fig. 2.2). They provide increased membrane surface and are principal sites of respiratory enzymes in bacteria. They are analogous to mitochondria of eukaryotes and are more prominent in Gram-positive bacteria. There are two types of mesosomes—septal and lateral. The septal mesosome is attached to bacterial chromosome and is involved in DNA segregation and in the formation of cross walls during binary fission.

Intracytoplasmic inclusions

Many species of bacteria produce cytoplasmic inclusion bodies which appear as round granules. They are not permanent or essential structures and may be absent under

certain conditions of growth. They are large polymeric complexes consisting of volutin (polyphosphates), lipid, glycogen, starch or sulphur. Generally, they are present in larger number when bacteria have access to an abundance of energy-yielding nutrients and diminish or disappear under conditions of energy source starvation.

Bacterial nucleus

The genetic information of a bacterial cell is contained in a single, circular, double-stranded molecule of DNA. It is often accompanied by a smaller extrachromosomal DNA known as **plasmid**. It is 1000 μm or more in length, about 1000 times the length of the cell. Therefore, it occurs tightly coiled like a skein of woollen thread. Since it is not bound to proteins, therefore, it does not stain like a eukaryotic chromosome. **Bacterial nucleus does not possess nuclear membrane, nucleolus, deoxyribonucleoprotein and does not divide by mitosis.**

Capsule and slime layer

Cell wall in many bacteria is enclosed by a protective gelatinous covering layer. If it is easily washed off and does not appear to be associated with the cell in any definite fashion it is referred to as a **slime layer**, on the other hand, if it appears as discrete, thickened gel around each cell, it is called a **capsule** (Figs 2.2 and 2.6). If capsule is too thin to be seen with light microscope, it is called **microcapsule**. In most species, it is made up of a complex polysaccharide (e.g. pneumococcus), though in some species its main constituent is polypeptide (e.g. anthrax bacillus). When slime forming bacteria are grown on a solid culture medium, the slime remains around the bacteria as a matrix in which they are embedded and its presence confers on growth a **mucoid** character.

Demonstration of capsule

- Capsule cannot be stained with ordinary stains like Gram staining.
- It can be visualized by suspending the organisms in India ink and observing microscopically the exclusion of the colloidal ink particles from the area around the cell that is occupied by the capsule.
- It may also be visualized by reaction with specific antibody, which causes a characteristic swelling of the capsule. It is known as **Quellung reaction**. This phenomenon is seen in and allows rapid identification of capsular serotypes of *Streptococcus pneumoniae*, *Neisseria meningitidis*, several groups of streptococci, *Klebsiella* and *Haemophilus influenzae*.

For demonstration of polypeptide capsule of *Bacillus anthracis* (McFadyean reaction) refer to Chapter 26.

Functions

- Capsules protect the bacteria from antibacterial agents such as lytic enzymes found in nature.
- They inhibit phagocytosis, thus contributing to the virulence of the bacteria.
- The capsular antigen is specific for bacteria, therefore, it can be used for identification and typing of bacteria.

Loss of capsule by mutation may render the bacterium avirulent. Bacteria tend to lose capsules on repeated sub-cultures *in vitro*.

Flagella

Flagella are long, hollow, helical filaments usually several times the length of the cell. They are 10–20 nm in diameter, 3–20 µm in length and are found on both Gram-positive and Gram-negative bacteria. Their number varies from 1 to 20 flagella per bacterial cell. They are organelles of locomotion.

Arrangement

There are four types of arrangement of flagella (Fig. 2.6):

1. **Monotrichous:** These organisms have a single polar flagellum.
2. **Lophotrichous:** They have a tuft of flagella at one pole.
3. **Amphitrichous:** They have single polar flagellum or tuft of flagella at both poles.
4. **Peritrichous:** Flagella are distributed all around the cell.

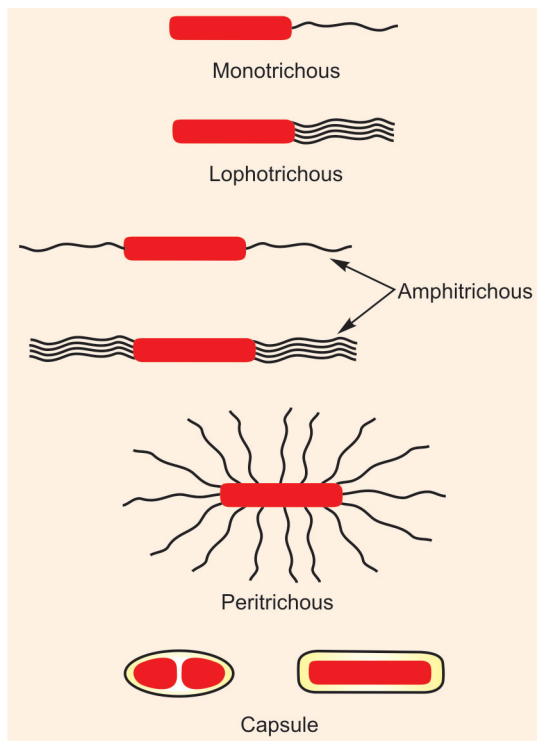


Fig. 2.6: Arrangement of flagella and capsule.

Flagella consist mainly of a protein called flagellin which belongs to the same chemical group as myosin—the contractile protein of muscle. Though, flagella of different genera of bacteria have the same chemical composition, they are antigenically different. Flagellar antigens induce specific antibodies in high titres. These antibodies are non-protective but are useful in serodiagnosis.

Demonstration of flagella

Flagella can be demonstrated by:

- Ordinary light microscope by special staining techniques in which their thickness is increased by mordanting.

- Dark-ground microscopy.
- Electron microscopy.

Indirect methods by which motility of bacteria can be demonstrated

- Occurrence of spreading growth in semisolid agar medium.
- On microscopic examination of wet films, motile bacteria are seen swimming in different directions across the field with darting (*V. cholerae*), very active (*Proteus* spp.), active (*Escherichia coli*), sluggish and tumbling (*Listeria monocytogenes*) motility. True motility should be differentiated from Brownian movement which is a rapid oscillation of the bacterium within a very limited area due to bombardment by the water molecules.

Structure of flagellum

The flagellum consists of three parts—the **filament**, the **hook** and the **basal body**. The basal body, anchored in the cytoplasmic membrane, comprises a rod and two or more sets of encircling rings. In Gram-negative bacteria four types of rings (M, S, P and L) are seen. Through ring M it attaches to the cytoplasmic membrane, ring S is located just above cytoplasmic membrane and through rings P and L it is attached to peptidoglycan and outer lipopolysaccharide membrane, respectively (Fig. 2.7). Rings P and L are absent in Gram-positive bacteria.

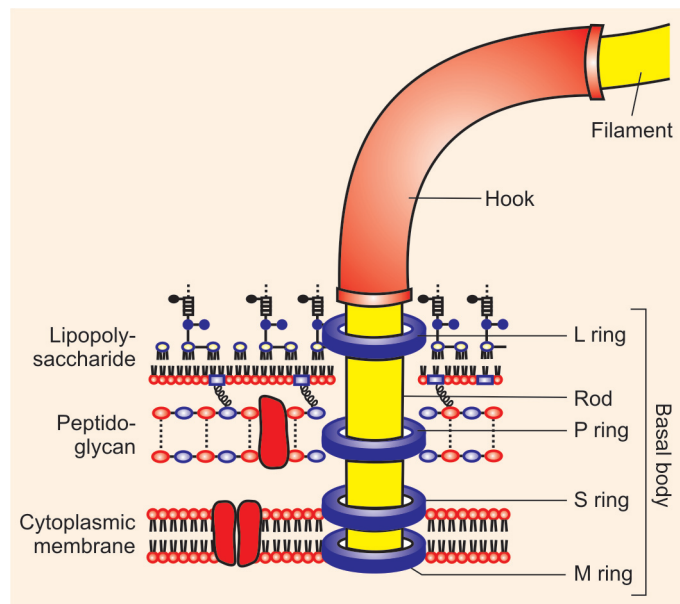


Fig. 2.7: Structure of flagellum.

Fimbriae or pili

They are hair-like surface appendages 1–1.5 µm in length and 4–8 nm in diameter. They are straighter, thinner and shorter than flagella (Fig. 2.2). They are present on many Gram-negative cells and provide a means for adherence to other cells, either bacterial or animal. They are an example of a class of surface structures termed **adhesins** that allow attachment of bacterial cell-to-cell surfaces (organelles of

adhesion). Therefore, they are very important for bacterial survival in an animal host. They occur in both flagellated and non-flagellated bacteria and are far more numerous than flagella. Each bacterium possesses 100–500 peritrichously-borne fimbriae. They can be seen by electron microscopy. They originate in the cytoplasmic membrane and are composed of self-aggregating protein monomers.

In stagnant liquid medium, the fimbriate bacteria grow attached together in the form of a pellicle that floats on the surface of the medium where the growth is greatly enhanced by the free supply of oxygen.

Certain bacteria possess specialized fimbriae or pili which are longer and thinner than the common type. These appear to be hollow and constitute conjugation tubes through which DNA is transferred from one organism to another during conjugation. They are determined by sex factors and are referred to as **sex pili** or conjugation pili.

Detection of fimbriae

- Electron microscopy.
- Haemagglutination: Most of the fimbriae can adhere to the red blood cells (RBCs) of many animal species. They bind very strongly to guinea pig, fowl, horse and pig; moderately strongly to human; and scarcely to ox RBCs. Therefore, a simple haemagglutination test can be used to determine whether a culture contains fimbriate bacilli or not.

Bacterial spore

Some species of bacteria (Gram-positive only), particularly those of the genera *Bacillus* and *Clostridium*, are capable of forming spores inside original cell. These spores can be released from original cell as free spores. Each bacterium forms one spore which on germination forms a single vegetative cell. Sporulation in bacteria, therefore, is a method of preservation and not of reproduction.

Sporulation

It develops from a portion of protoplasm near one end of the cell. This part of the bacterial cell is known as **forespore** and the remaining part as **sporangium** (Fig. 2.8). Bacterial DNA replicates and partitions into two halves and one of them, which is equivalent to one genome of the cell, is incorporated into forespore. A transverse septum derived from the cytoplasmic membrane is then formed by a process of invagination which divides forespore and sporangium. The forespore is, subsequently, completely encircled by dividing septum as a double layered membrane.

The two spore membranes now engage in active synthesis of various layers of the spore. The inner layer becomes the **inner membrane**. Between the two layers is laid **spore cortex** and outer layer is transformed into **spore coat** which consists of several layers. In some species from outer layer

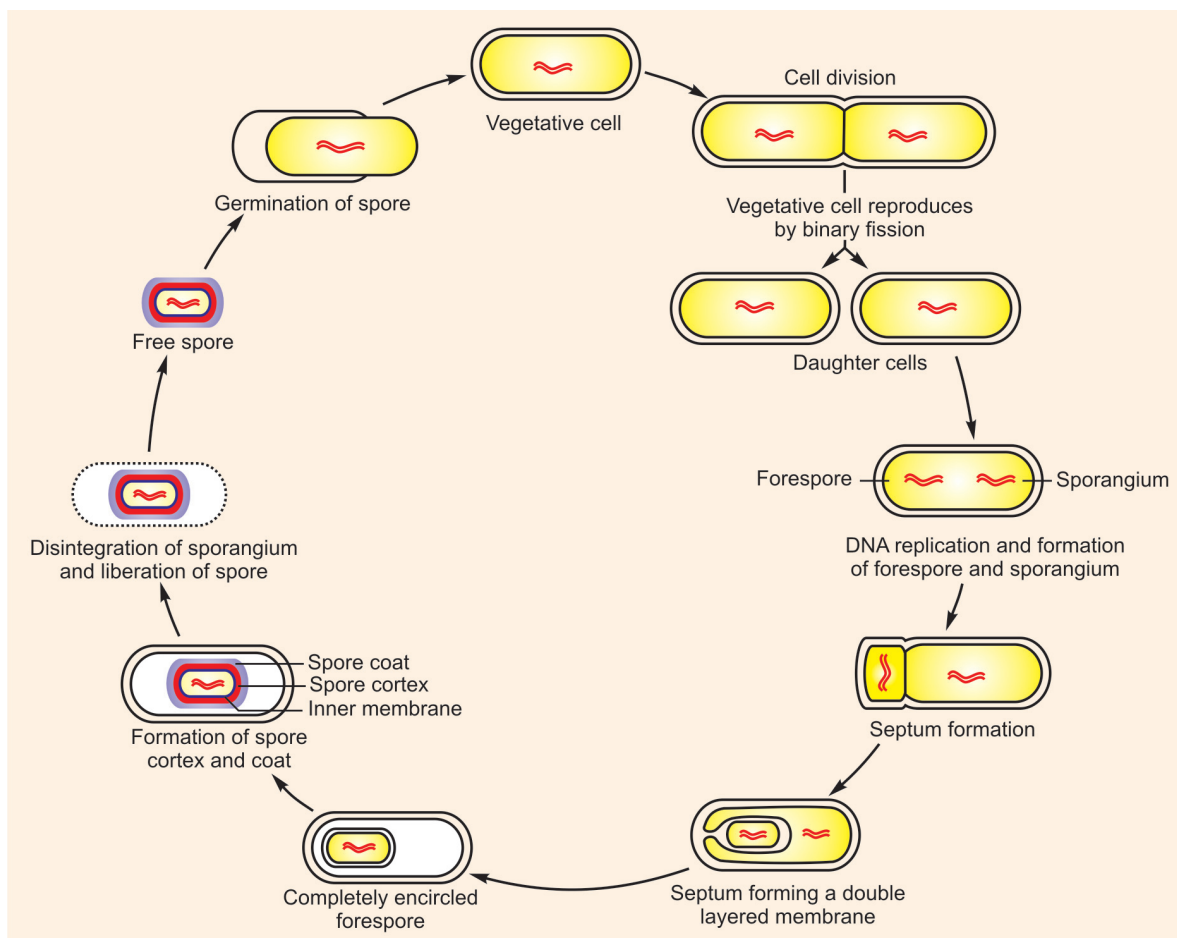


Fig. 2.8: Morphological events in sporulation.

also develops **exosporium**, which bears ridges and folds (Fig. 2.9). Finally, sporangium disintegrates and the spore is freed. Mesosomes appear to play a role in the development of spores and may be involved in the compartmentation of the spore's share of the nuclear material.

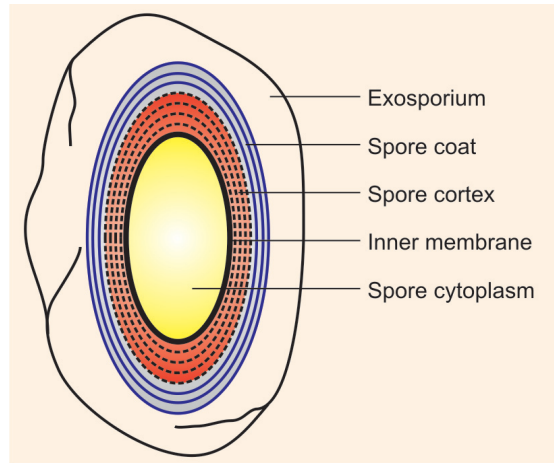


Fig. 2.9: Bacterial spore.

Shape and position

The spores may be round, oval or elongated occupying a terminal, subterminal or central position. They may be narrower than the width of the bacilli or broader and bulging (Fig. 2.10).

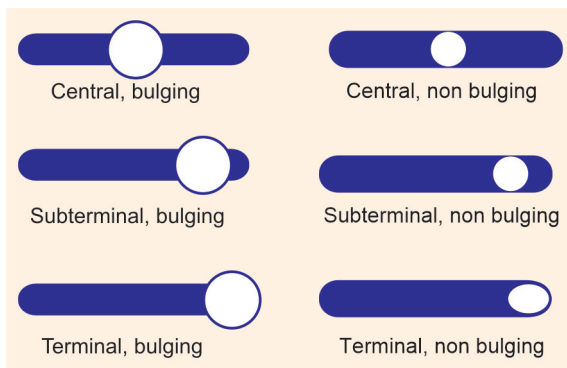


Fig. 2.10: Types of spores.

Resistance

Spores can remain dormant for many years. They are extremely resistant to chemical and physical agents. Their killing requires moist heat at 100–120°C for 10 minutes while vegetative cells can be killed by heating at 60°C for 10 minutes. Marked resistance of the spores is due to:

- The impermeability of their cortex and outer coat,
- Their high content of calcium and dipicolinic acid,
- Their low content of water, and
- Their very low metabolic and enzymatic activity.

Germination

Spores are able to **germinate** when the external conditions become favourable to growth by access to moisture and

nutrients. It swells after which the spore coat ruptures, and a new vegetative cell grows out.

Demonstration

- In unstained preparations, the spore is recognized within the parent cell by its greater refractility. In simple stains like Gram it remains unstained and appears as a clear space within the stained cell protoplasm (Fig. 2.11).
- They are slightly acid-fast and may be demonstrated by modified Ziehl-Neelsen staining.

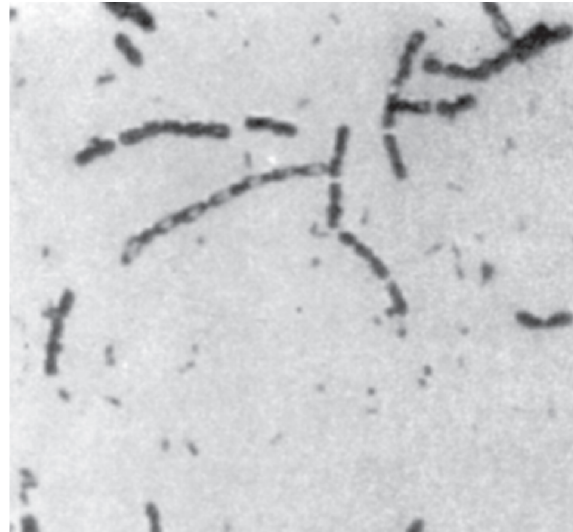


Fig. 2.11: Gram-stained smear of *Bacillus subtilis* showing Gram-positive bacilli in chains with spores which appear as unstained areas within the bacilli ($\times 1,000$).

L-forms of bacteria

L-forms (after Lister Institute, London) of bacteria are cell wall deficient bacteria derived by variation, usually in the laboratory, from bacteria of normal morphology. They are stable in the sense that special conditions of culture, such as presence of penicillin, are not required to prevent their reversion to the parental bacterial forms. They lack regular size and shape. They may be spherical or disc-like and measure 0.1–20 μm in diameter.

Cultural characteristics

L-forms are difficult to grow and usually require a medium that is solidified with agar as well as having the right osmotic strength. L-forms are produced more readily with penicillin than with lysozyme.

Colonies of L-forms of bacteria on agar medium show a characteristic '**fried-egg**' appearance with a dark thick centre, where many of the organisms embed themselves and grow within the agar, and a lighter periphery consisting of organisms lying on the surface of the agar. In liquid medium they grow in the form of clumps. Some L-forms are capable of reverting to normal bacillary forms upon removal of the inducing stimulus. Other L-forms are, however, stable and never revert. Presence of residual peptidoglycan is essential for reversion. It acts as a primer in its own biosynthesis.

KEY POINTS

- Prokaryotes such as **bacteria are simple cells** with no internal membranes or organelles.
- **Eukaryotes have a nucleus and organelles** such as mitochondria, and complex **internal membranes** (e.g. fungi and human cells).
- **Structures external** to cell wall of bacteria are **flagella, pili** or **fimbriae, capsule** and **slime layer**.
- Flagella are used for movement, pili for adhesion, and capsules protect the bacteria from antibacterial agents such as lytic enzymes and inhibit phagocytosis thus contributing to the virulence of bacteria.
- **Peptidoglycan is present in the cell wall of both Gram-positive and Gram-negative bacteria**, but is thicker in the former and gives rigidity and shape to the organism.
- Peptidoglycan comprises long chains of *N*-acetylglucosamine and *N*-acetylmuramic acid; a set of identical tetrapeptide side chains attached to *N*-acetylmuramic acid, and a set of identical pentapeptide cross-bridges.
- In the outer membrane of Gram-negative bacteria are embedded various large molecules like outer membrane protein and porin protein. Large antibiotic molecules penetrate outer membrane relatively slowly, which accounts for the **relatively high antibiotic resistance of Gram-negative bacteria**.
- Lipopolysaccharide (LPS) is the outermost layer of outer membrane of Gram-negative (but not Gram-positive) bacteria; **LPS is endotoxin** and, therefore, Gram-positive bacteria do not produce endotoxin.
- **Bacterial cytoplasm contains** chromosomal nuclear material, ribosomes, mesosomes and inclusions/storage granules.
- **Sporulation** is a response to starvation in *Bacillus* spp. and *Clostridium* spp. They are a means of survival, not reproduction.



Important Questions

1. Differentiate between prokaryotes and eukaryotes in a tabulated form.
2. Draw a labelled diagram of a bacterial cell.
3. Describe the cell wall of bacteria.
4. Differentiate between flagella and fimbria in a tabulated form.
5. Write short notes on:

(a) Capsule	(b) Intracytoplasmic inclusions in bacteria
(c) Bacterial spore	(d) Mesosomes



Multiple Choice Questions

1. Which of the following bacteria is cell wall deficient?
 - (a) *Mycoplasma*.
 - (b) *Treponema*.
 - (c) *Staphylococcus*.
 - (d) *Klebsiella*.
2. Chinese letter arrangement is characteristic of:
 - (a) *Mycobacterium tuberculosis*.
 - (b) *Bacillus anthracis*.
 - (c) *Corynebacterium diphtheriae*.
 - (d) *Clostridium tetani*.
3. Peptidoglycan is major constituent of cell wall of:
 - (a) Gram-positive bacteria.
 - (b) Gram-negative bacteria.
 - (c) fungi.
 - (d) protozoa.
4. Lipopolysaccharide is a constituent of cell wall in:
 - (a) Gram-positive bacteria.
 - (b) Gram-negative bacteria.
 - (c) fungi.
 - (d) protozoa.
5. Sterols are present in the cytoplasmic membrane of:

(a) <i>Mycoplasma</i> .	(b) <i>Bacillus</i> .
(c) <i>Clostridium</i> .	(d) <i>Proteus</i> .
6. Mesosomes of bacteria are analogous to:
 - (a) mitochondria of eukaryotes.
 - (b) lysosomes of eukaryotes.
 - (c) Golgi apparatus of eukaryotes.
 - (d) nucleolus of eukaryotes.
7. When flagella are distributed all around the bacterial cell, the arrangement is known as:
 - (a) monotrichous.
 - (b) lophotrichous.
 - (c) amphitrichous.
 - (d) peritrichous.
8. Ultraviolet light is used in:
 - (a) fluorescence microscope.
 - (b) dark-ground microscope.
 - (c) phase-contrast microscope.
 - (d) interference microscope.
9. Polymers *N*-acetylglucosamine and *N*-acetylmuramic acid are found in:
 - (a) cell membrane.
 - (b) cell wall.
 - (c) outer membrane.
 - (d) capsule.

10. Teichoic acid is present in:
 - (a) Gram-negative organisms.
 - (b) Gram-positive organisms.
 - (c) *Mycoplasma*.
 - (d) *Rickettsia*.
11. Cell wall deficient forms of Gram-positive bacteria are called:
 - (a) spheroplasts.
 - (b) trophozoites.
 - (c) cysts.
 - (d) protoplasts.
12. Sedimentation coefficient of bacterial ribosomes is:
 - (a) 50S.
 - (b) 60S.
 - (c) 70S.
 - (d) 80S.
13. Bacterial structure concerned with respiration is:
 - (a) mesosomes.
 - (b) ribosomes.
 - (c) mitochondria.
 - (d) Golgi apparatus.
14. Which of the following bacteria has peritrichous flagella?
 - (a) *Vibrio cholerae*.
 - (b) *Proteus*.
 - (c) *Pseudomonas*.
 - (d) *Shigella*.
15. Which of the following bacteria has a single polar flagellum?
 - (a) *Vibrio*.
 - (b) *Salmonella*.
 - (c) *Proteus*.
 - (d) *Citrobacter*.
16. Which of the following bacterial structures is involved in attachment to cell surface?
 - (a) Flagella.
 - (b) Fimbria.
 - (c) Capsule.
 - (d) Mesosomes.
17. Which of the following are eukaryotes?
 - (a) Fungi.
 - (b) Bacteria.
 - (c) Chlamydiae.
 - (d) Mycoplasmas.
18. Which of the following structures is found in Gram-negative and not in Gram-positive bacteria?
 - (a) Capsule.
 - (b) Cytoplasmic membrane.
 - (c) Ribosomes.
 - (d) Outer membrane.
19. Which of the following structures is found in Gram-positive and not in Gram-negative bacteria?
 - (a) Capsule.
 - (b) Cytoplasmic membrane.
 - (c) Spores.
 - (d) Outer membrane.
20. Which of the following is a distinctive component of the cell wall of Gram-negative bacteria?
 - (a) Capsule.
 - (b) Lipopolysaccharide.
 - (c) Peptidoglycan.
 - (d) Cytoplasmic membrane.
21. When determining distances and sizes, the smallest unit of measure is:
 - (a) centimetre.
 - (b) millimetre.
 - (c) micrometre.
 - (d) nanometre.
22. What function does a condenser serve in light microscope?
 - (a) focuses the light onto our eyes.
 - (b) focuses the light rays on the sample.
 - (c) increases light intensity.
 - (d) reduces glare.
23. Bacterial capsules are generally viewed by:
 - (a) Albert staining.
 - (b) Ziehl-Neelsen staining.
 - (c) Negative staining.
 - (d) All of the above.
24. Fimbriae present on the outer surface of bacteria are used for:
 - (a) inhibition of phagocytosis.
 - (b) bacterial motility.
 - (c) adherence to surfaces.
 - (d) none of the above.



Answers

- | | | | | | | | | | | | |
|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| 1. a | 2. c | 3. a | 4. b | 5. a | 6. a | 7. d | 8. a | 9. b | 10. b | 11. d | 12. c |
| 13. a | 14. b | 15. a | 16. b | 17. a | 18. d | 19. c | 20. b | 21. d | 22. b | 23. c | 24. c |

Growth and Nutrition of Bacteria

BACTERIAL GROWTH

Bacteria reproduce by a process called binary fission, in which a parent cell divides to form a progeny of two cells. This results in a logarithmic growth rate—one bacterium will produce 16 bacteria after four generations.

Generation time

The time required for a bacterium to give rise to two daughter cells is known as **generation time**. Under constant conditions, the generation time for any organism is quite reproducible, but differs greatly among different bacteria. The fastest growing bacteria have generation time of 15–20 minutes under optimum growth conditions. Many bacteria, however, have generation times of hours or even days. In *Escherichia coli* it is 20 minutes, in tubercle bacilli it is 14–15 hours and in lepra bacilli it is 12–13 days.

Batch culture

When bacteria are grown in liquid medium, multiplication is arrested after a few cell divisions due to depletion of nutrients and/or accumulation of toxic products. This is known as batch culture.

Continuous culture

By use of special devices like chemostat or turbidostat in which nutrients are replaced and bacteria are removed continuously it is possible to maintain continuous culture of bacteria for industrial and research purposes.

When pathogenic bacteria multiply in the host tissues the situation is intermediate between batch culture and continuous culture because they get inexhaustible source of nutrients but they have to face host defence mechanisms.

In liquid media, growth of bacteria is diffuse and on solid media they form colonies. Each colony consists of a clone of cells derived from a single parent cell. Bacteria in a culture medium or clinical specimen can be counted by two methods:

1. Total count

This is total number of bacteria present in a specimen irrespective of whether they are living or dead. This is done

by counting the bacteria under microscope using counting chamber and by comparing the growth with standard opacity tubes.

2. Viable count

This measures only viable (living) cells which are capable of growing and producing a colony on a suitable medium.

BACTERIAL GROWTH CURVE

When a bacterium is inoculated into a suitable culture medium and incubated, its growth follows a characteristic course. If both total and viable counts are made at different intervals and plotted in relation to time, then a characteristic growth curve is obtained. A typical growth curve contains four major phases (Fig. 3.1).

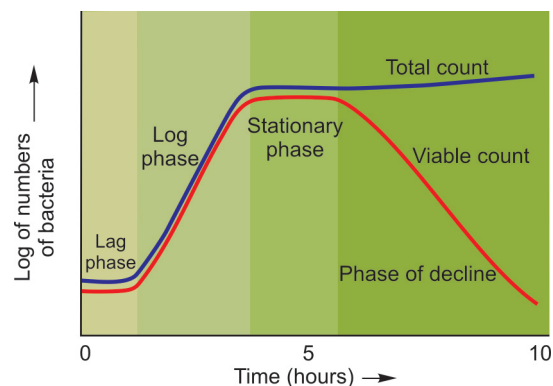


Fig. 3.1: Bacterial growth curve.

1. Lag phase

When bacteria are seeded into fresh medium, multiplication usually does not begin immediately. The period between inoculation and beginning of multiplication is known as lag phase. **During this period the organisms adapt themselves to growth in fresh medium and increase in size and metabolic activity. Therefore, lag phase is regarded as a period not of rest but of intense metabolic activity.**

The duration of lag phase varies with the species, nature of culture medium, temperature of incubation, etc. Depending

on the growth medium, the lag phase may be short or very long. For example, if a culture in a rich growth medium that supplies most of the cells' requirements is inoculated into a poor medium that requires the cells to make most of their own amino acids and vitamins, the lag phase will be very long. The cells must activate the metabolic pathways for amino acid and vitamin synthesis and must make enough of these nutrients to begin active growth. In contrast, the cells that are inoculated from one medium to a fresh tube of the same medium may show virtually no lag phase, since they need not change their metabolism.

2. Log or exponential growth phase

During this phase the bacteria are multiplying at their maximum rate and their number increases exponentially or by geometric progression with time. If logarithm of bacterial count is plotted against time a straight line is obtained. **In the log phase, the bacterial cells are smaller and stain uniformly.** Exponential phase is of limited duration because of:

- Exhaustion of nutrients,
- Accumulation of toxic metabolic end products,
- Rise in cell density,
- Change in pH, and
- Decrease in oxygen tension (in case of aerobic organisms).

3. Stationary phase

Due to above reasons exponential growth slows down and the bacterial population enters the stationary phase in which the number of viable cells remains constant. There is almost a balance between the bacterial reproduction and bacterial death. **During this phase, bacteria become Gram-variable, show irregular staining and spores start forming in spore-forming bacteria.**

4. Phase of decline

Stationary phase is followed eventually by the phase of decline because rate of death exceeds the rate of reproduction and the number of viable cells declines. **Finally, after a variable period, all the cells die and culture becomes sterile.**

Bacterial nutrition

The growth of microorganisms depends upon an adequate supply of suitable nutrients, pH, oxygen and temperature. They require the elements present in their chemical composition. Nutrients must provide these elements in a metabolically accessible form. All bacteria have three major nutritional needs for growth:

1. A source of carbon for making cellular constituents.
2. A source of nitrogen for making proteins.
3. A source of energy (ATP) in order to synthesize macromolecules and maintain essential chemical gradients across their membranes.

Nutritional requirements for growth

Bacteria are divided into two basic groups according to how they meet their nutritional needs:

1. Autotrophs

Some organisms possess considerable synthetic power, therefore, they can utilize very simple inorganic compounds, such as carbon dioxide as carbon source and ammonium salts as nitrogen source. These are known as autotrophs or lithotrophs. Autotrophs obtain energy either photosynthetically (phototrophs) or by oxidation of inorganic compounds (chemotrophs).

2. Heterotrophs

Bacteria that are unable to synthesize their own metabolites and depend on preformed organic compounds are known as heterotrophs. They require an organic source of carbon, such as glucose, and obtain energy by oxidizing or fermenting organic substances. Often, the same substance (for example, glucose) is used as both the carbon source and energy source. Some bacteria require certain organic compounds in minute quantities. These are known as growth factors or bacterial vitamins.

All bacteria that inhabit the human body fall into the heterotrophic group. Within this group, however, nutritional needs vary greatly. Some bacteria like *E. coli* can grow under a wide range of conditions while others like *Neisseria gonorrhoeae* and *Haemophilus influenzae* are exacting and restrictive in their requirements.

CULTURE MEDIA

Numerous culture media have been devised. The original media used by Louis Pasteur were liquids such as urine or meat broth. Liquid media have many disadvantages. Bacteria growing in these media may not exhibit specific characteristics for their identification. With liquid media it is difficult to isolate different types of bacteria from mixed populations. However, liquid media are used for obtaining bacterial growth from blood or water when large volumes have to be used as inoculum, for preparing bulk cultures for antigens and vaccines, and for preparation of inoculum for biochemical reactions and antibiotic susceptibility testing.

In 1881, Robert Koch described means of cultivating bacteria on solid media. First he used as his growth medium pieces of potato, then 2.5–5.0% gelatin to prepare solid media fortifying them with 1% meat extract as an essential ingredient. But gelatin is not satisfactory as it liquefies at 24°C (incubation temperature for most pathogenic bacteria is 37°C) and many proteolytic bacteria liquefy gelatin. At the suggestion of Anglina Hesse, the American wife of his assistant, he substituted agar-agar in place of gelatin as solidifying agent for the media. She had used it to solidify broths in her kitchen.

Agar-agar or '**agar**' for short is prepared from a variety of seaweeds; the product is clarified, dried and supplied as a powder. It does not add to the nutritive properties of medium and is not affected by the growth of bacteria. The exact concentration to be used may require some adjustment according to the batch of agar. A concentration of 1–2% usually yields a suitable gel. In preparing agar media, the

appropriate amount of agar powder is added to the liquid medium and dissolved by placing the mixture in a steamer at 100°C for 1 hour or longer. Most agars dissolve to give a clear solution but sometimes it is necessary to filter off particulate impurities.

The melting and solidifying points of agar solutions are not the same. At the concentrations normally used, most bacteriological agars melt at about 95°C and solidify only when cooled to about 42°C. Agar can be added to any nutrient liquid medium if the advantages of a solid medium are desired. Most of the culture media are sterilized by autoclaving at 121°C for 15 minutes. Nutrients that are damaged by autoclaving are sterilized separately by filtration, etc. The sterilized agar base is then melted in the steamer and cooled to about 45–50°C followed by addition of heat-labile ingredients, but once these are added the medium must at once be poured into Petri dishes because it cannot be remelted without damaging the heat-sensitive ingredients.

Another important ingredient of common media is **peptone**. It consists of water-soluble products obtained from lean meat or other protein material such as heart muscle, casein, fibrin or soya flour, usually by digestion with the proteolytic enzymes pepsin, trypsin or papain. Its constituents are peptones, proteoses, amino acids, a variety of inorganic salts including phosphates, potassium and magnesium, and certain accessory growth factors such as nicotinic acid and riboflavin. Special brands of peptone such as neopeptone and proteose peptone are available for special use.

Other common ingredients of the culture media include casein hydrolysate, meat extract, yeast extract, malt extract, blood and serum.

While bacteria grow diffusely in liquid media, they produce discrete visible growth on solid media in Petri dishes. If a mixed culture is inoculated in suitable dilution on solid medium, different bacteria form well-separated colonies, which are clones of cells originating from a single bacterial cell. On solid media, bacteria have distinct colony morphology and exhibit many other characteristic features such as pigment production or haemolysis.

Types of culture media

Culture media have been classified in many ways:

1. Solid, semisolid and liquid.
2. Simple (basal), complex, synthetic, defined, semidefined and special media. Special media are further divided into enriched, selective, enrichment, indicator or differential, sugar media and transport media.
3. Aerobic media and anaerobic media.

Basal media

These include peptone water, and nutrient broths which form the basis of most media used in the study of the common pathogenic bacteria.

Meat extract broth is most commonly used nutrient broth. It consists of peptone, meat extract, sodium chloride and water. It can be made solid by addition of 1–2% agar. If the concentration of agar is reduced to 0.2–0.5%, it is called

semisolid agar. If its concentration is raised to 6%, it is called **hard agar**. In semisolid agar the motile organisms show growth in entire medium, and on surface of hard agar swarming of *Proteus* is inhibited.

Enriched media

These are prepared to meet the nutritional requirements of fastidious organisms by addition of substances such as blood, serum and egg to a basal medium. Important examples of enriched media are blood agar for isolation of *Streptococcus*, chocolate agar for isolation of *Neisseria* and *Haemophilus*, and Loeffler's serum slope for the isolation of *Corynebacterium diphtheriae*.

Selective media

When a substance is added to a solid medium which inhibits the growth of unwanted bacteria but permits the growth of wanted bacteria in the form of colonies, it is known as selective medium. Important examples of this type of media are MacConkey agar for *E. coli*, deoxycholate citrate agar (DCA) for *Salmonella* and *Shigella*, Wilson and Blair's medium for *Salmonella*, Lowenstein-Jensen medium for *Mycobacterium tuberculosis*, and blood tellurite agar medium for isolation of *Corynebacterium diphtheriae*.

Enrichment media

When a substance is added to a liquid medium which inhibits the growth of unwanted bacteria and favours the growth of wanted bacteria it is known as enrichment medium. Important examples of this type of media are tetrathionate broth and selenite F broth for *Salmonella* and *Shigella*, and alkaline peptone water for *Vibrio cholerae*.

Indicator media or differential media

When a substance is added into a medium which would produce a visible change in the medium following the growth of a particular organism, it is designated as indicator or differential medium. For example, MacConkey medium contains lactose and neutral red. Lactose fermenting organisms, after growth on this medium, produce acid and in acidic pH neutral red becomes red in colour. Thus, *E. coli* which is lactose fermenter produces red or pink colonies on this medium.

Christensen's medium contains urea and phenol red. When urease producing organisms like *Proteus* and *Klebsiella* grow on this medium, urea is split up into ammonia and carbon dioxide. Ammonia makes the medium alkaline and in alkaline pH the medium becomes pink in colour (in alkaline pH phenol red is pink in colour). Enriched media can also be differential on the basis of certain growth characteristics evident on the medium. Blood agar is considered both an enriched and differential medium because it differentiates organisms based on whether they are α -haemolytic, β -haemolytic or non-haemolytic.

Transport or holding media

When a clinical sample is being transported from the hospital to the laboratory, delicate organisms like *Neisseria*

gonorrhoeae may not survive or the normal flora (*E. coli*) may overgrow pathogenic flora (*Salmonella*, *Shigella* and *V. cholerae*). Transport media maintain the viability of microorganisms present in a specimen without supporting the growth of any organism. These maintain the organisms in a state of suspended animation so that no organism overgrows another or dies out. These media typically contain only buffers and salts. They lack carbon, nitrogen and organic growth factors, hence do not facilitate microbial multiplication. Stuart's transport medium and Amies transport medium are examples of transport media.

Storage media

Bacteria are best preserved and stored by lyophilization. But for preservation and storage for a few months or so, they can be stab inoculated on semisolid agar or on Dorset egg medium followed by incubation. When growth appears they can be stored in refrigerator.

Defined synthetic media

These media are prepared from pure chemical substances, therefore, their exact composition is known. These are used for research purposes.

Sugar media

For the identification of most of the organisms, sugar fermentation reactions are carried out. Glucose, lactose, sucrose and mannitol are widely used sugars. For the preparation of sugar media, 1% of the concerned sugar is added to peptone water with a suitable indicator. Durham's tube (a small tube) is kept inverted in the tube containing this medium to detect gas production. For fastidious organisms like *C. diphtheriae* and pneumococci, Hiss's serum sugar media are used.

Anaerobic media

For the growth of anaerobes, the media used contain reducing substances. These include thioglycollate broth and cooked meat broth. The sterile muscle tissue, in cooked meat broth, contains reducing substances, particularly glutathione, which permit the growth of many strict anaerobes. In addition to its reducing effect, the meat provides a variety of nutritional substances for bacterial growth. In this medium, saccharolytic clostridia rapidly produce acid and gas but do not digest the meat. The cultures may have slight sour smell and the meat is often reddened. The proteolytic clostridia produce blackening of the meat, decomposing it and reducing it in volume with the formation of foul-smelling products.

ENVIRONMENTAL FACTORS INFLUENCING GROWTH

Oxygen

On the basis of the influence of oxygen on growth and viability, the bacteria are divided into two categories— aerobes and anaerobes:

- **Aerobes** require oxygen for their growth. They may be obligate aerobes like *Pseudomonas aeruginosa* which can

grow only in the presence of oxygen, and facultative anaerobes. The latter are ordinarily aerobes, but they can also grow in the absence of oxygen though less abundantly.

- **Anaerobes**, on the other hand, are organisms that do not require oxygen for life and reproduction. In addition, oxygen's direct toxic effect may prohibit the growth of these organisms in environments in which oxygen is present. They may be obligate anaerobes such as *Clostridium tetani* which cannot grow even in the presence of traces of oxygen, and **microaerophilic** such as *C. perfringens* which can grow under microaerophilic conditions.

Carbon dioxide

Some organisms such as *Brucella abortus* require extra CO₂ in the air in which they are grown and others such as pneumococci and gonococci grow better in air supplemented with 5–10% CO₂ (**capnophilic**).

Temperature

Each bacterium multiplies best within a restricted temperature range. For most of the pathogenic bacteria optimum temperature for growth is 37°C (our body temperature) with upper and lower temperature limits of 40–50°C and 15–20°C, respectively. The organisms with optimum temperatures of 37°C, less than 20°C and 55–80°C are known as mesophiles, psychrophiles and thermophiles, respectively.

Moisture and desiccation

Moisture is very essential for the growth of bacteria because 80% of their body weight is made up of water. However, the effect of drying varies in different organisms. For example *Treponema pallidum*, gonococci and human immunodeficiency virus die quickly after drying while tubercle bacilli and staphylococci may survive drying for several weeks. However, bacterial spores can survive for several years, and drying in cold and vacuum (**lyophilization**) is a method for preservation of bacteria and viruses.

pH

Like other living organisms, microorganisms are very susceptible to changes in the acidity or alkalinity of the surrounding medium. Most of the medically important bacteria can grow at neutral or slightly alkaline pH (7.2–7.6). Some bacteria like lactobacilli and cholera vibrios grow at acidic and alkaline pH, respectively.

Light and other radiations

Darkness provides a favourable condition for growth and viability of bacteria. Ultraviolet rays from direct sunlight or a mercury lamp are bactericidal. Bacteria are also killed by ionizing radiations. Photochromogenic mycobacteria form pigment only on exposure to light.

Osmotic effect

Because of the mechanical strength of the cell wall, bacteria are more tolerant to osmotic variation, therefore, they can

grow in media with widely varying contents of salt, sugar and other solutes. Sudden exposure of bacteria to solutions of high salt concentration may cause **plasmolysis**. This is due to osmotic withdrawal of water leading to shrinkage of protoplast and its retraction from the cell wall. This occurs more readily in Gram-negative than Gram-positive bacteria. On the other hand, sudden transfer of bacteria from concentrated solution to distilled water may cause **plasmolysis** due to excessive osmotic imbibition of water leading to swelling and bursting of cell.

Mechanical and sonic stresses

In spite of the mechanical strength of the cell wall, bacteria can be ruptured and killed by vigorous shaking with glass beads and ultrasonic vibrations.

CULTURE METHODS

The methods of bacterial culture used in the clinical laboratory include streak culture, lawn culture, stroke culture, stab culture, pour-plate culture, shake culture and liquid culture.

Streak culture

This method is routinely employed for the isolation of bacteria in pure culture from clinical specimens. A platinum or nichrome wire loop of 2–4 mm diameter is used. The loop is first sterilized in the bunsen flame by making it red hot and cooled by touching on uninoculated part of the medium. Then a loopful of the specimen is smeared thoroughly over area *A* (Fig. 3.2), on the surface of a well dried plate, to give a well-inoculum or 'well'. The loop is re-sterilized and drawn from the well in 2 or 3 parallel lines onto the fresh surface of the medium (*B*). This process is repeated as shown (*C*, *D* and *E*), care being taken to sterilize the loop, and cool it on unseeded medium, between each sequence. On incubation, growth may be confluent at the site 'well', but becomes progressively thinner, and well separated colonies are obtained over the final series of streaks.

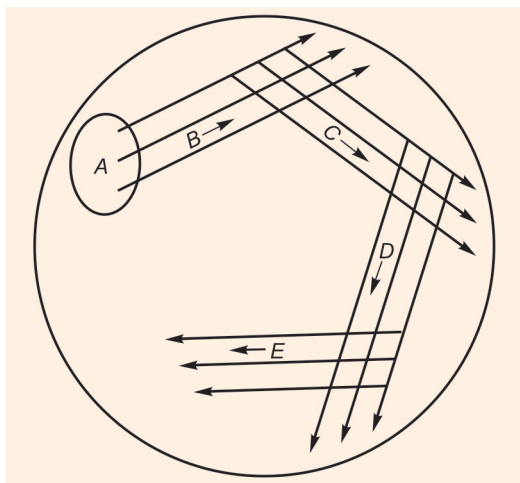


Fig. 3.2: Streak culture.

Lawn culture

Lawn cultures are prepared by flooding the surface of the plate with a liquid culture or suspension of bacteria and pipetting off the excess inoculum or by applying a swab soaked in the bacterial culture or suspension. After incubation, lawn culture provides a uniform surface growth. It is useful for antibiotic susceptibility testing by disc diffusion method and bacteriophage typing.

Stroke culture

Stroke culture is made in tubes containing agar slope or slant. Slopes are seeded by lightly smearing the surface of agar with loop in a zig-zag pattern taking care not to cut the agar. It is used for obtaining pure growth for slide agglutination and other diagnostic tests.

Stab culture

Stab cultures in solid media (nutrient gelatin or glucose agar) are inoculated by plunging the charged wire into the centre of the medium and withdrawing it in the same line to avoid splitting the medium. These are employed mainly for demonstration of gelatin liquefaction and for the maintenance of stock cultures.

Pour-plate culture

This method is used for counting the number of living bacteria or groups of bacteria in a liquid culture or suspension. Prepare serial 10-fold dilutions of the bacterial suspension over a range (6–9 tubes) ensuring that one dilution will contain between 50–500 viable bacteria/ml (number which can be accurately counted). Starting with the greatest dilution, pipette 1 ml amounts of each dilution into each of three 9 cm Petri dishes. Then pour into each dish about 10 ml of clear nutrient agar, melted, and cooled at 45–50°C. Mix well by rapidly moving the plate for about 10 seconds. Allow the agar to set and incubate at 37°C for 48 hours. After incubation, colonies will be seen well distributed throughout the depth of the medium and can be enumerated using colony counters. Count the colonies in three plates containing 50–500 colonies/plate. Multiply the average number/plate by the dilution factor to obtain the viable count/ml in the original suspension.

Shake culture

It is made by melting nutrient agar in a test tube, cooling it to 45°C and inoculating it while molten from a liquid medium with a drop from a capillary pipette. Withdraw the pipette, replace the cap or plug and discard the pipette into disinfectant. Mix the contents of the tube by rotation between the palms of the hands before the agar solidifies and incubate it at 37°C for 24 hours and look for the growth of the organisms.

Liquid culture

Liquid cultures in tubes, bottles or flasks may be inoculated by touching with a charged loop or by adding the inoculum with pipettes or syringes. Large inocula can be employed in

liquid cultures and hence this method is adopted for blood culture and for sterility tests, where the concentration of bacteria in inocula are expected to be small. Liquid cultures are also preferred when large yields are desired.

AEROBIC CULTURE

For cultivation of aerobes the incubation is done in an incubator under normal atmospheric condition. The temperature of incubation for most of the human pathogenic bacteria is 37°C. To prevent drying of the medium when prolonged incubation is necessary, as in the cultivation of the tubercle bacilli, screw-capped bottles should be used instead of test tubes or plates.

CULTURE IN AN ATMOSPHERE WITH ADDED CARBON DIOXIDE

Some organisms such as *Brucella abortus* and capnophilic streptococci, require extra CO₂ in the air in which they are grown and others, such as the pneumococcus and gonococcus grow better in air supplemented with 5–10% CO₂. For this, CO₂ jars are used. The required amount of air is withdrawn with a vacuum pump and replaced with CO₂ from a cylinder. CO₂ incubators which provide a predetermined and regulated amount of CO₂ in a suitably humid atmosphere are commercially available. Screw caps on containers of liquid media must not be tight and should preferably be replaced by a closure that allows entry of CO₂.

CULTURE IN MICROAEROPHILIC ATMOSPHERE

Microorganisms like *Campylobacter*, *Helicobacter pylori* and *Actinomyces israelii* are microaerophilic. Culture of such organisms is done by an evacuation replacement method with 5% O₂, 10% CO₂ and 85% N₂.

ANAEROBIC CULTURE

Anaerobic culture methods

A variety of methods are available for the culture of anaerobic organisms in the clinical laboratory. Exclusion of oxygen from the medium is the simplest method, and is effected by growing the organisms within the culture medium such as freshly steamed liquid media containing reducing agents such as glucose, ascorbic acid, cysteine, sodium thioglycollate and cooked meat pieces.

Cooked meat broth (CMB), an original medium known as 'Robertson's bullock-heart medium' has a special place in anaerobic bacteriology; and **thioglycollate broth** and its modifications are also very useful. CMB is suitable for growing anaerobes in air and also for the preservation of stock cultures of aerobic organisms. The inoculum is introduced deep in the medium in contact with the meat. Cooked meat pieces are placed in 30 ml bottles to a depth of about 2.5 cm and covered with about 15 ml broth.

Anaerobic Jars

When an oxygen-free or anaerobic atmosphere is required for obtaining surface growths of anaerobes, anaerobic jars provide the method of choice. The most reliable and widely used anaerobic jar is the **McIntosh-Fildes anaerobic jar**. It is a cylindrical vessel made of glass or metal with a metal lid which is held firmly in place by a clamp (Fig. 3.3). The lid has two tubes with taps, one acting as gas inlet and the other as the outlet. On its undersurface it carries a gauze sachet carrying alumina pellets coated with palladium. It acts as a room temperature catalyst for the conversion of hydrogen and oxygen into water. It acts as a catalyst, as long as the sachet is kept dry.

Inoculated culture plates are placed inside the jar and the lid clamped tight. The outlet tube is connected to a vacuum pump and the air inside is evacuated. The outlet tap is then closed and the inlet tube connected to a hydrogen supply. Hydrogen is drawn in rapidly. As soon as this inrush of gas has ceased, the inlet tap is also closed. After about 5 minutes inlet tap is again opened. There occurs again an immediate inrush of hydrogen since the catalyst creates a reduced pressure within the jar due to the conversion of hydrogen and left over oxygen into water. If there is no inrush of hydrogen, it means the catalyst is inactive and must be replaced. The jar is left connected to the hydrogen supply for about 5 minutes, then the inlet tap is closed and the jar is placed in the incubator, catalysis will continue until all the oxygen in the jar has been used up.

The **GasPak** is now the method of choice for preparing anaerobic jar. The GasPak is commercially available as a disposable envelope containing chemicals which generate hydrogen and carbon dioxide on the addition of water. After the inoculated plates are kept in the jar, the GasPak envelope with water added, is placed inside and the lid screwed tight. Hydrogen and carbon dioxide are liberated and the presence of a cold catalyst in the envelope permits the combination of hydrogen and oxygen to produce an anaerobic environment. The outstanding feature of the GasPak system is the

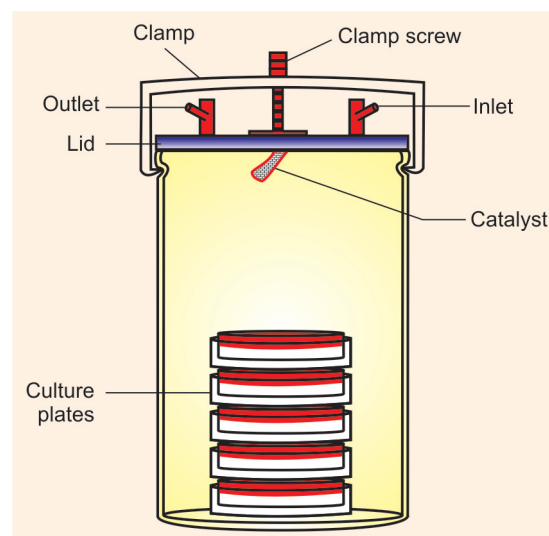


Fig. 3.3: Anaerobic jar.

disposable gas generator envelope, which does away with the need for a vacuum pump and cylinders of compressed gas; the operation of the jar is consequently very quick and simple. As the standard GasPak jar is not evacuated before use a relatively large volume of water is formed during catalysis.

An **indicator** should be used for verifying the anaerobic condition in the jar. Methylene blue is generally used for this purpose. When it is placed in an anaerobic environment, it is reduced from its coloured oxidized form to a colourless reduced leuco compound.

In addition to, or instead of, using a chemical indicator, some workers include in the jar a plate inoculated with a known strict anaerobe such as *Clostridium tetani* or *Bacteroides fragilis*, and a strict aerobe, such as *Pseudomonas aeruginosa*. This method is quite reliable if the indicator anaerobe grows and the aerobe does not.

The major disadvantage of any anaerobic jar system is that the plates have to be removed from the jar to be examined. This, of course, exposes the colonies to oxygen, which is especially hazardous to the anaerobes during their first 48 hours of growth. For this reason, a suitable holding system should always be used in conjunction with anaerobic jars, placed in an oxygen-free holding system, removed one by one for rapid microscopic examination of colonies, and then quickly returned to the holding system. Plates should never remain in room air on the open bench.

Anaerobic chamber

This is an ideal anaerobic incubation system, which provides oxygen-free environment for inoculating media and incubating cultures. Identification and susceptibility tests can also be performed in anaerobic chambers.

Anaerobic chambers may be fitted with airtight rubber gloves to insert hands and manipulate specimens, plates, tubes or they may be gloveless where airtight rubber sleeves fit tightly against user's bare forearms. All anaerobic chambers contain a catalyst, dessicant, H₂ gas (5–10%), CO₂ gas (5–10%), N₂ gas (80–90%) and an indicator.

Anaerobic bags or pouches

These bags are available commercially and one or two inoculated plates are placed into a bag and an oxygen removal system is activated and the bag is sealed and incubated. Plates can be examined for growth without removing the plates from bag, thus without exposing the colonies to oxygen. But as with anaerobic jar, plates must be removed from the bags in order to work with the colonies at the bench. These bags are also useful in transport of biopsy specimen for anaerobic cultures.

Incubation

Inoculated plates should be incubated at 37°C for at least 48 hours, and reincubated for another 2–4 days to allow slow-growing organisms (certain species of *Actinomyces* and *Eubacterium*) to form colonies.

KEY POINTS

- Bacteria **reproduce** by **binary fission** leading to logarithmic growth of cell numbers; generation time of bacteria varies from minutes to hours or days.
- Bacterial **growth** in laboratory media can be divided into a **lag** phase, **log** phase, **stationary** phase and phase of **decline**.
- Depending on their oxygen requirements, bacteria can be divided into **obligate aerobes**, **facultative anaerobes**, **obligate anaerobes** and **microaerophiles**.
- For **cultivation and identification** of bacteria, basal media, enriched media, selective media, enrichment media, differential media, etc. are used.
- Depending upon the expected organisms, the inoculated media are incubated in **aerobic culture**, **culture in an atmosphere with added carbon dioxide**, **culture in microaerophilic atmosphere** and **anaerobic culture**.



Important Questions

1. Describe bacterial growth curve.
2. Define generation time of bacteria. Discuss briefly batch culture and continuous culture.
3. What are culture media? Classify and discuss them in brief.
4. Write short notes on:
 - (a) Basal media
 - (b) Enriched media
 - (c) Selective media
 - (d) Enrichment media
 - (e) Transport media
 - (f) Differential media
 - (g) Sugar media
 - (h) Synthetic media
5. Discuss in detail anaerobic culture methods.



Multiple Choice Questions

1. Generation time of *Escherichia coli* is:
(a) 20 seconds. (b) 20 minutes.
(c) 20 hours. (d) 20 days.
2. The period between inoculation of bacteria in a culture medium and beginning of multiplication is known as:
(a) lag phase. (b) log phase.
(c) stationary phase. (d) decline phase.
3. Peptone water and nutrient broth are:
(a) basal media.
(b) enriched media.
(c) selective media.
(d) enrichment media.
4. When a substance is added to a solid medium which inhibits the growth of unwanted bacteria but permits the growth of wanted bacteria, it is known as:
(a) selective medium.
(b) enrichment medium.
(c) enriched medium.
(d) differential medium.
5. When a substance is added to a liquid medium which inhibits the growth of unwanted bacteria and favours the growth of wanted bacteria, it is known as:
(a) selective medium.
(b) enrichment medium.
(c) enriched medium.
(d) differential medium.
6. MacConkey medium is an example of:
(a) transport medium. (b) differential medium.
(c) enrichment medium. (d) enriched medium.
7. Stuart's transport medium is used for transport of specimen containing:
(a) *Salmonella*.
(b) *Vibrio cholerae*.
(c) *Neisseria gonorrhoeae*.
(d) *Shigella*.
8. Bacteria, whose optimum temperature for growth is 37°C, are known as:
(a) mesophiles. (b) psychrophiles.
(c) thermophiles. (d) None of the above.
9. Which of the following bacteria dies quickly after drying?
(a) *Mycobacterium tuberculosis*.
(b) *Staphylococcus aureus*.
(c) *Pseudomonas aeruginosa*.
(d) *Treponema pallidum*.
10. Which of the following bacteria can grow in alkaline pH?
(a) Lactobacilli.
(b) *Vibrio cholerae*.
(c) *Salmonella*.
(d) *Shigella*.
11. Which of the following bacteria can grow in acidic pH?
(a) Lactobacilli.
(b) *Vibrio cholerae*.
(c) *Salmonella*.
(d) *Shigella*.
12. All bacteria that inhabit the human body are:
(a) autotrophs.
(b) heterotrophs.
(c) phototrophs.
(d) chemolithotrophs.
13. Which of the following is **not** a differential medium?
(a) Christensen's medium.
(b) MacConkey medium.
(c) Blood tellurite agar.
(d) Mueller-Hinton agar.
14. Chocolate agar is an example of which of the following media?
(a) Non-selective media.
(b) Selective media.
(c) Differential media.
(d) Enriched media.



Answers

1. b 2. a 3. a 4. a 5. b 6. b 7. c 8. a 9. d 10. b 11. a 12. b
13. d 14. d

Collection of Specimens, Identification of Bacteria and Taxonomy

Competency achievement: The student after reading the chapter should be able to:

MI1.2: Perform and identify different causative agents of infectious diseases by Gram stain and Ziehl-Neelsen stain.

MI1.3: Describe the epidemiological basis of common infectious diseases.

MI6.1: Describe the etiopathogenesis, laboratory diagnosis and prevention of infections of upper and lower respiratory tract.

MI6.2: Identify the common etiologic agents of upper respiratory tract infections (Gram staining).

MI6.3: Identify the common etiologic agents of lower respiratory tract infections (Gram stain and Ziehl-Neelsen stain).

MI7.1: Describe the etiopathogenesis and discuss laboratory diagnosis of infections of genitourinary system.

MI7.2: Describe the etiopathogenesis and discuss the laboratory diagnosis of sexually transmitted infections. Describe the preventive measures.

MI8.9: Discuss the appropriate method of collection of specimen in the performance of laboratory tests in the detection of microbial agents causing infectious disease.

IM1.22: Assist and demonstrate the proper technique in collecting specimen for blood culture.

IM4.13: Perform and interpret a sputum Gram stain.

IM4.14: Perform and interpret a sputum Ziehl-Neelsen stain.

IM4.19: Assist in the collection of blood and wound cultures.

IM25.9: Assist in the collection of blood and other specimen cultures.

PE34.11: Perform Ziehl-Neelsen staining.

The laboratory diagnosis of an infectious disease begins with the collection of a clinical specimen. Proper collection of an appropriate clinical specimen is the first step in obtaining

an accurate laboratory diagnosis of an infectious disease. A poorly collected specimen not only may result in failure to recover important microorganisms, but may also lead to incorrect or even harmful therapy if treatment is directed towards a commensal or contaminant.

The quality of a clinical microbiological report is directly related to the quality of the specimen on which it is based. Care in obtaining a proper specimen and its prompt submission to the laboratory are essential. In general, specimens of frank pus, wound exudate or excised tissue sent in sterile containers are preferable to swab which is relatively inefficient sampling device.

General rules for collection and transportation of specimen

- Apply strict aseptic techniques throughout the procedure.
- Wash hands before and after the collection.
- Collect the specimen before the administration of antimicrobial agents.
- Prevent contamination of the specimen with externally present organisms or normal flora of the body.
- Collect the specimen at the appropriate phase of disease.
- Collect the specimen from the actual infection site.
- Collect adequate quantity for the desired tests.
- Collect the specimen aseptically in a sterile and appropriate container.
- Close the container tightly so that its contents do not leak during transportation.
- Ensure that the outside of the specimen container is clean and uncontaminated.
- Label the container appropriately and complete the requisition form.
- Immediately transport the specimen to the laboratory.

Criteria for rejection of specimens

- Missing or inadequate identifications.
- Incomplete forms.
- Leaking container or blood stained containers.
- Specimens collected in an inappropriate container.
- Haemolysed blood sample.
- Insufficient quantity.

- Dried-up specimen.
- Contamination suspected.
- Specimen collected in formalin.
- Inappropriate transport or storage.

Blood for culture

- Using a pressure cuff, locate a suitable vein in the arm.
- Wearing sterile gloves thoroughly disinfect the venepuncture site as follows:
 - Using spirit (70% ethanol) swab, cleanse an area about 50 mm in diameter. Allow to air-dry.
 - Using 2% tincture of iodine and a circular action, swab the area beginning at the point where the needle will enter the vein. Allow the iodine to dry on skin for at least 1 minute.
- Lift the tape or remove the protective cover from the top of culture bottle. Wipe the top of the bottle using spirit swab.
- Using a sterile syringe and needle withdraw 5 ml of blood from an adult and 1 ml from a young child.
- Insert the needle through a hole in the cap and through rubber or plastic liner of the bottle cap, and dispense 5 ml of blood from an adult and 1 ml from a young child into the culture medium bottles (Fig. 4.1a), respectively. Cap must not be removed for introduction of the blood. In adults large quantity (5 ml) of blood is required since the number of organisms in the blood particularly in mild and recovering cases may be quite small, even as few as one per ml. As blood's natural bactericidal or bacteriostatic action may interfere with the growth of any bacteria present, this effect is annulled by diluting (inoculating) 5 ml of blood in an adult and 1 ml of blood in a young child in 50 ml and 10 ml of the medium (10-fold dilution), respectively. **Organisms causing bacteraemia in young children are usually present in sufficient concentration to be detected in small volume (1 ml) of blood.**
- Using a fresh spirit swab wipe the top of the culture bottle and replace the tape or protective cover. Without delay,

mix the blood with the broth. Blood must not be allowed to clot in the culture medium because any bacteria will become trapped in the clot.

- Clearly label the bottle with the name and number of the patient, and the date and time of collection.
- As soon as possible, incubate the inoculated medium.
- Collect blood during early stage of disease since the number of bacteria is higher in acute and early stage of disease.
- In the absence of antibiotic administration, 99% culture positivity can be seen with three blood cultures.
- Transport the specimen to the laboratory. If delay in transportation is expected keep in incubator or at room temperature. **Do not refrigerate.**

Blood for serological tests

For serological tests, collect about 5 ml of blood to ensure there will be enough serum for all the tests that may be required. Immediately transfer the blood from the syringe into a dry stoppered sterile tube or bottle (without anti-coagulant) and allow to clot. When the serum has separated, pipette it off into a sterile tube.

Urine

- Mid-stream urine sample is collected after giving proper instructions to the patient.
 - (a) Clean the genitalia properly. (In case of male, retract the prepuce, clean it with sterile normal saline. In case of female, wash perineum and periurethral area with soap and water. Separate apart labia with fingers of one hand).
 - (b) Collect a 'clean-catch' mid-stream urine sample in a sterile container (Fig. 4.1b).
- Transport immediately to the laboratory. If a delay of more than 1–2 hours is unavoidable, refrigerate at 4°C.
- In catheterized patients, do not collect urine from collection bag or after opening the closed drainage. Clean the area over the collecting tube and puncture with the help of a sterile needle and syringe and draw out the sample.
- Suprapubic aspiration under aseptic condition may be done in infants.

Cerebrospinal fluid (CSF)

Cerebrospinal fluid must be collected by an experienced physician. Rigorous aseptic precautions must be observed to prevent the introduction of infection into the central nervous system. The fluid is usually collected from the arachnoid space.

- Skin between fourth and fifth vertebrae is decontaminated with spirit and tincture of iodine.
- A sterile wide-bore needle is inserted between fourth and fifth lumbar vertebrae and the CSF is allowed to drip into a sterile dry container (Fig. 4.1c).
- Only 3–5 ml of fluid should be collected, because the removal of a larger volume may lead to headache.
- Immediately deliver the sample with a request form to the laboratory.

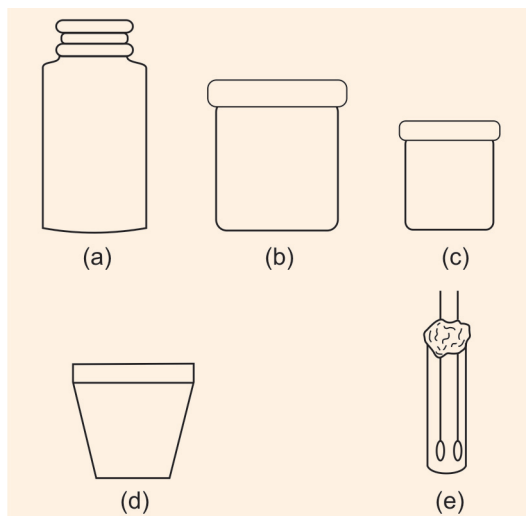


Fig. 4.1: (a) McCartney bottle; (b) Urine/stool container; (c) CSF container; (d) Sputum container; (e) Throat swabs.

- CSF must be examined without delay and the results of the tests reported to the medical officer as soon as they become available. If delay in processing is inevitable then store it at 37° C. *Do not refrigerate.*
- The fluid should be handled with special care because a lumbar puncture is required to collect the specimen.

Sputum

- Collect the sputum in a wide-mouthed container, which is preferably disposable, made up of transparent thin plastic, unbreakable and leak-proof (Fig. 4.1d).
- Ask the patient to rinse the mouth with plain water and then inhale deeply 2–3 times, cough up deeply and spit in the sputum container by bringing it close to the mouth. If the patient has difficulty in coughing sputum, postural drainage, and appropriate physiotherapy often cause exudate to move in the bronchi and stimulate productive coughing.
- Make sure the sputum sample is of good quality and not just the saliva. A good sputum sample is thick, purulent and sufficient in amount (2–3 ml).
- Sputum sample may be refrigerated for up to 3–4 hours.

Throat swab

- Two swabs should be collected, one for smear and other for culture (Fig. 4.1e).
- Depress the tongue with a tongue blade.
- Swab the inflamed area of the throat, pharynx or tonsils with a sterile swab taking care to collect the pus or piece of membrane.
- Take care not to contaminate the swab with saliva.

Pus and other discharge

- Do not apply antiseptic before collection.
- Clean with normal saline.
- In case of discharge, 1–2 ml of sample is collected in a sterile vial.
- If swabs are to be collected, then two swabs, in a sterile container, should be collected (one for direct microscopic examination and the other for culture).

Bone marrow

- Decontaminate the skin, overlying the site from where specimen is to be collected, with spirit and tincture of iodine.
- Aspirate 1 ml or more of bone marrow by sterile percutaneous aspiration with bone marrow aspiration needle.
- Collect in a sterile screw-capped tube.
- Immediately transport to the laboratory.

Stool

- Should be collected in early stage of disease and prior to treatment with antimicrobials.
- Do not collect the specimen from bed pan.
- Should not be contaminated with urine.

- Collect about a spoonful of the specimen, especially that which contains mucus, pus or blood, into a clean, dry, leakproof container (Fig. 4.1b).
- If possible, submit more than one specimen on different days.
- The fresh stool specimen must be processed within 1–2 hours of passage.
- If delay is unavoidable then store it at 2–8°C.

Rectal swab

- Collect the swab only if stool collection is not possible.
- Insert swab at least 2.5 cm beyond the anal sphincter, so that it enters the rectum.
- Rotate it once before withdrawing.
- Transport in Cary-Blair or other transport medium.

IDENTIFICATION OF BACTERIA

Identification of the isolate is carried out by examination of stained and unstained smears of the morbid material, isolation in pure culture on appropriate culture media, study of macroscopic (colonial characters) and microscopic morphology of the isolate, and biochemical characters.

Microscopic morphology of bacteria, appearance of bacterial colonies on solid media and appearance of bacterial growth in liquid media are given in Tables 4.1, 4.2 and 4.3, respectively.

Unstained wet film

An unstained wet film or **hanging drop preparation** is examined under light microscope for observation of motility, and an unstained wet film may be examined under **dark-ground microscope** for demonstration of motility of spirochaetes. Presence of *Treponema pallidum*, with characteristic spiral shape and motility, in exudate from a chancre is sufficient for presumptive diagnosis of syphilis.

Table 4.1: Microscopic morphology of bacteria

- **Shape:** Cocci, bacilli, coccobacilli, comma-shaped or filamentous.
- **Size:** Length and breadth, and diameter in μm .
- **Sides:** Parallel, convex, concave or irregular.
- **Ends:** Rounded, truncate, concave or pointed.
- **Arrangement:** Pairs, chains, groups of four or eight, clumps. Bacilli may be arranged at random, in short or long chains, in Chinese letter patterns, as palisades or in bundles. Vibrios may be single or in S forms.
- **Motility:** Non-motile, sluggishly motile, tumbling motility, actively motile, very actively motile or darting motility.
- **Flagella:** Without flagella (atrichate), monotrichate, lophotrichate, amphitrichate or peritrichate.
- **Spores:** These, when present, may be oval, spherical or ellipsoidal. These may be of the same width or wider than that of bacillary body. The spores may be equatorial, subterminal or terminal.
- **Capsules:** Present or absent.
- **Staining:** Even, irregular, bipolar, beaded, barred, presence of metachromatic granules, Gram-positive, Gram-negative, acid-fast, non-acid-fast.

Table 4.2: Appearance of bacterial colonies on solid media

- **Size:** In mm.
- **Shape:** Circular, irregular.
- **Elevation:** Flat, convex, concave, umbonate or umblicate.
- **Edges:** Entire, undulate, crenated or fimbriate.
- **Colour:** White, buff, pink, etc.
- **Structure:** Translucent, transparent or opaque.
- **Consistency:** Membranous, friable, butyrous or viscid.
- **Emulsifiability:** Emulsifiable or autoagglutinable.
- **Degree of growth:** Scanty, moderate or profuse.
- **Haemolysis:** Type of haemolysis (α , β or non-haemolytic) produced on blood agar.

Table 4.3: Appearance of bacterial growth in liquid media

- **Degree of growth:** Scanty, moderate or profuse.
- **Turbidity:** Present or absent. If present, uniform, granular or flocculent.
- **Deposit:** Present or absent. If present, powdery, granular or membranous.
- **Surface pellicle:** Present or absent.

Staining techniques

A number of staining techniques for the identification of bacteria are available. Of these, Gram stain and Ziehl-Neelsen stain are most important. A Gram-stained smear shows the Gram reaction, size, shape and grouping pattern of bacteria, absence or presence of spores, their shape, size and intracellular position. Presence of Gram-negative diplococci inside the polymorphs in cerebrospinal fluid (CSF) and urethral discharge gives the provisional diagnosis of meningococcal meningitis and gonorrhoea, respectively. With Ziehl-Neelsen staining, it is possible to identify tubercle bacilli and atypical mycobacteria, lepra bacilli, and *Nocardia*. They resist decolourization with 20%, 5% and 1% sulphuric acid, respectively.

Gram stain

Gram stain is named after Hans Christian Gram, a bacteriologist from Denmark who developed the technique in the 1880s. This staining method is most frequently used in diagnostic bacteriology.

Method

- Prepare smear from clinical specimen, culture smear of colony or broth culture on a clean glass slide by spreading thinly on the slide.
- Allow it to dry in air and fix it by passing the dried slide 3–4 times through the flame quickly with smear side facing up.
- Cover the smear with crystal violet (primary stain) for one minute. Other pararosaniline dyes such as methyl violet or gentian violet may also be used as primary stain.
- Pour Gram's iodine over the slide for one minute.
- Drain off the iodine.
- Decolourize with alcohol or acetone for 10–30 seconds.
- Wash the smear with water.
- Counterstain with dilute carbol fuchsin or safranin for 30 seconds.
- Wash thoroughly with water; blot and dry in air.
- Examine under oil-immersion lens.

On the basis of their reaction to the Gram stain, bacteria can be divided into two groups, i.e. Gram-positive and Gram-negative. Both Gram-positive and Gram-negative bacteria take up violet colour with pararosaniline dyes. After treatment with decolourizing agent, Gram-positive bacteria retain this dye and violet colour while Gram-negative lose the dye and become colourless. They then take up counterstain and appear red in colour.

The Gram stain is useful in two ways:

1. In the identification of many bacteria.
2. In influencing the choice of antibiotic.

Mechanism of Gram staining

The exact mechanism of Gram reaction is not known. It may, however, be attributed to:

- Gram-positive bacteria have a more acidic protoplasm, which may account for their retaining the basic primary dye more strongly than Gram-negative bacteria.
- The violet basic dye and the iodine form a dye-iodine complex inside both Gram-positive and Gram-negative bacteria but during alcohol or acetone wash, cell membranes (outer membrane of cell wall and cytoplasmic membrane) are dissolved. However, dye-iodine complex is retained in Gram-positive cells by the thick peptidoglycan mesh, whereas it is readily washed out through the very thin peptidoglycan layer remaining in Gram-negative cells after both membranes have been dissolved.
- Gram-positive organisms that have lost cell wall integrity because of antibiotic treatment, old age, or action of autolytic enzymes may allow crystal violet to wash out with the decolourizing step and may appear Gram-variable, with some cells staining pink and others staining violet. Host cells, such as red and white blood cells, allow the crystal violet stain to wash out with decolourization and appear red.

Ziehl-Neelsen stain

Next to Gram stain, this is the method most frequently used in diagnostic bacteriology. It is of value in distinguishing a few bacterial species, e.g. tubercle bacilli, non-tuberculous mycobacteria, lepra bacilli and *Nocardia* from all others. Tubercle bacilli, non-tuberculous mycobacteria and lepra bacilli are relatively impermeable to simple stains but when stained with hot concentrated carbol fuchsin, subsequently resist decolourization with 20%, 20%, 5% and 1% sulphuric acid, respectively. Decolourized non-acid-fast organisms are counterstained with methylene blue. However, *Nocardia* which resists decolourization with 1% sulphuric acid, can be easily stained with Gram stain and is Gram-positive.

Modified Ziehl-Neelsen staining is used for detection of oocysts of *Cryptosporidium*, *Cytoisosporea*, *Cyclospora* and microsporidia.

Method

- Cover the slide with filtered carbol fuchsin and heat until steam rises. Allow the preparation to stain for 5 minutes, heat being applied at intervals to keep the stain hot. The stain must not be allowed to evaporate and dry on the slide. If necessary, pour more carbol fuchsin to keep the whole slide covered with carbol fuchsin.
- Wash with water.
- The stained smear is decolourized with 20% sulphuric acid and washed with water. This step is repeated till the film is only very faintly pink.
- Wash the slide well with water.
- Counterstain it with 2% methylene blue for 15–20 seconds.
- Wash, blot dry the smear and examine under oil-immersion lens.

Result

Acid-fast bacilli stain bright red, while the tissue cells and other organisms are stained blue.

Principle of acid-fastness

Acid-fastness has been attributed to the high content of lipids, fatty acids and higher alcohols found in acid-fast bacteria. Of the lipids, mycolic acid, a high molecular weight hydroxy acid wax containing carboxyl groups, is most important because it is acid-fast even in free state.

Albert's stain

This method is useful for identification of *Corynebacterium diphtheriae*.

Method

- Prepare the film, dry in air, and fix by heat.
- Cover slide with Albert's stain and allow to act for 3–5 minutes.
- Wash in water and blot dry.
- Cover the slide with Albert's iodine, allow to act for one minute.
- Wash in water, blot dry and examine under oil-immersion lens.

Result

The granules and the protoplasm of *C. diphtheriae* are bluish-black and green, respectively.

Other stains

A number of other staining procedures are available. They include:

- Simple stains such as methylene blue and basic fuchsin. They impart same colour to all organisms and are used only for colour contrast.
- Too thin bacteria may be rendered visible by light microscope by **silver impregnation method** which thickens the bacteria. This method is used for demonstration of spirochaetes and flagella.
- In case of **negative staining**, bacteria (spirochaetes) or fungi (*Cryptococcus*) are mixed with India ink or nigrosin

that provide a uniform coloured background against which the unstained organisms can be seen. Bacterial capsules which do not take simple stains can be seen by negative staining.

- Special staining techniques for demonstration of spores, flagella, cell walls and capsules of bacteria are also available.

Differential identification characteristics

Accurate identification can be accomplished by isolation of bacteria in pure form followed by study of colonial morphology, examination of stained smear, biochemical reactions, antigenic structure, serotyping, biotyping, bacteriocin typing, phage typing and animal pathogenicity. Clinical material is inoculated onto a solid medium (nutrient agar, blood agar or MacConkey agar) in such a way, so as to ensure isolated discrete colonies. A colony represents a clone of descendants of a single bacterium, therefore, pure growth.

Enriched, enrichment, selective and differential media, depending upon the organism suspected, are employed. Selective growth conditions, i.e. presence or absence of oxygen and presence of CO₂, etc. are also employed keeping in view the organisms suspected. The culture plates are incubated at optimum temperature. Most of the pathogenic bacteria grow best at 37°C.

Biochemical reactions

A large number of biochemical tests can be employed for the identification of different bacteria. These include:

Indole production

Certain bacteria which possess enzyme **tryptophanase**, degrade amino acid tryptophan to indole, pyruvic acid and ammonia. Indole production is detected by inoculating the test organism into peptone water and incubating it at 37°C for 48–96 hours. Then add 0.5 ml of Kovac's reagent and shake gently. A **red colour** in the alcohol layer indicates a positive reaction. Kovac's reagent consists of:

Paradimethylaminobenzaldehyde	10 g
Amyl or isoamyl alcohol	150 ml
Concentrated hydrochloric acid	50 ml

Dissolve aldehyde in alcohol and slowly add the acid and store in refrigerator. Shake gently before use. Indole is extracted from the medium by amyl or isoamyl alcohol and forms red coloured ring by forming a red coloured complex with paradimethylaminobenzaldehyde. Negative test will show yellow coloured ring (colour of Kovac's reagent).

Methyl red (MR) test

This test detects the production of sufficient acid by fermentation of glucose, so that pH of the medium falls and it is maintained below 4.5. Inoculate the test organism in glucose phosphate broth and incubate at 37°C for 2–5 days. Then add 5 drops of 0.04% solution of methyl red, mix well and read the result immediately. Positive tests are bright red (indicating a low pH) and negative are yellow. If the test is negative after 2 days repeat it after 5 days.

Voges-Proskauer (VP) test for acetoin production

Many bacteria ferment carbohydrates with the production of acetyl methyl carbinol (acetoin). In the presence of potassium hydroxide and atmospheric oxygen, acetoin is converted to diacetyl, and α -naphthol serves as a catalyst to form a red complex. This test is usually done in conjunction with the methyl red test. *An organism of the family Enterobacteriaceae is usually either methyl red positive and Voges-Proskauer negative or methyl red negative and Voges-Proskauer positive.*

Inoculate test organism in glucose phosphate broth and incubate at 37°C for 48 hours. Then add 1 ml potassium hydroxide and 3 ml of 5% solution of α -naphthol in absolute alcohol. A positive reaction is indicated by the development of **pink colour** in 2–5 minutes and **crimson** in 30 minutes.

Citrate utilization

This test is used to study the ability of an organism to utilize citrate as a sole source of carbon for the growth. Liquid (Koser's) and solid (Simmon's) media containing citrate as a sole source of carbon can be used. A part of colony is picked up with a straight wire and inoculated into either of these media. The ability of an organism to utilize citrate as a sole source of carbon is detected by the **production of turbidity** (due to growth) in liquid medium. Solid medium also contains bromothymol blue as indicator, therefore, on the solid medium the **appearance of growth and blue colour** is positive, and original green colour and no growth is negative.

The blue colour is due to the alkaline pH that results from utilization of citrate. It turns the indicator in the medium from **green to blue**. It is important to keep the inoculum light, since dead organisms can be a source of carbon, producing a false positive reaction.

Indole, MR, VP and citrate tests are done in routine for the classification of Gram-negative enteric bacteria. They are commonly referred to as **IMViC tests**.

Sugar fermentation

The ability of an organism to ferment various sugars is tested by inoculation of the test organism in different sugar media containing Andrade's indicator. Glucose, mannitol, lactose and sucrose are mostly used sugars. A small inverted tube (**Durham's tube**) completely filled with liquid and containing no air bubbles is usually included in each culture tube. Production of acid is indicated by the change in the colour of the medium to red or pink, and gas, if produced, collects in Durham's tube.

Nitrate reduction

This test detects the production of enzyme nitrate reductase which reduces nitrate to nitrite. *All the organisms of the family Enterobacteriaceae are positive for this test.* Inoculate test organism in 5 ml medium containing potassium nitrate, peptone and distilled water. Incubate it at 37°C for 96 hours. Then add 0.1 ml test reagent which consists of equal volumes of 0.8% sulphanilic acid and 0.5% α -naphthylamine in 5 N acetic acid mixed just before use. A **red colour** developing

within a few minutes indicates the presence of nitrite and hence the ability of test organism to reduce nitrate to nitrite.

If no colour develops, this may indicate that nitrate has not been reduced (a true negative reaction) or that nitrate has been reduced beyond nitrite to nitrogen gas (N_2), nitric oxide (NO), or nitrous oxide (N_2O). Because the test reagents detect only nitrites, the latter process would lead to a false negative reading. Thus, it is necessary to add a small quantity of zinc dust to all negative reactions. Zinc ions reduce nitrate to nitrite, and the development of a red colour after adding zinc dust indicates the presence of residual nitrates and confirms a true negative reaction.

Urease test

This test detects the ability of an organism to produce urease enzyme. The test organism is inoculated on the entire slope of Christensen's medium which contains urea and phenol red indicator in addition to other constituents including agar. It is incubated at 37°C and examined after 4 hours and after overnight incubation. Development of **purple-pink** colour indicates production of urease. The latter in the presence of water converts urea into ammonia and carbon dioxide. Ammonia makes the medium alkaline and phenol red indicator changes to purple-pink in colour.

Hydrogen sulphide production

Some organisms produce hydrogen sulphide from sulphur containing amino acids. It may be detected by suspending strips of filter paper impregnated with lead acetate between the cotton plug and the tube. Blackening of the paper indicates hydrogen sulphide production. It has variable sensitivity. When cultured in media containing lead acetate or ferric ammonium citrate or ferrous acetate they turn them black or brown. This method is more sensitive than lead acetate strip method.

Potassium cyanide test

This tests the ability of an organism to grow in the presence of potassium cyanide. Inoculate buffered peptone water medium, containing 1 in 13,000 concentration of potassium cyanide, with test organism. Incubate at 37°C for 24–48 hours. Development of **turbidity** in the medium indicates the ability of the organism to grow in the presence of potassium cyanide.

Catalase production

The enzyme catalase mediates the breakdown of hydrogen peroxide into oxygen and water. This principle is used for the detection of catalase enzyme in a bacterial isolate.

Put a loopful of 10% hydrogen peroxide on colonies of the test organism on nutrient agar. Alternatively, pick up a few colonies of the test organism with platinum loop from nutrient agar plate and dip it in a drop of 10% hydrogen peroxide on a clean glass slide. The production of **gas bubbles** from the culture indicates a positive reaction. A false positive result may be obtained if the growth is picked up from medium containing catalase, e.g. blood agar or if an iron wire loop is used.

Oxidase test

This test depends on the presence, in bacteria, of certain oxidases that catalyze the oxidation of reduced tetramethyl-*p*-phenylene-diamine dihydrochloride (oxidase reagent) by molecular oxygen. Put a drop of freshly prepared 1% solution of oxidase reagent on a piece of filter paper. Then rub a few colonies of test organism on it. If it is oxidase-positive, it will produce a **deep purple** colour within 10 seconds. Alternatively, pour oxidase reagent over the colonies of the test organism on the culture plate. The colonies of oxidase-positive organisms rapidly develop a deep purple colour.

Various species of *Neisseria*, *Pseudomonas*, *Aeromonas*, *Vibrio*, *Alcaligenes* and *Campylobacter* are oxidase-positive. All the members of the family Enterobacteriaceae are oxidase-negative.

Motility test

The motility test medium has agar concentration of 0.4% or less, to allow free spread of organisms. Inoculation is done by a single stab into the medium. After overnight incubation, movement away from the stab line or a hazy appearance throughout the medium indicates a motile organism.

Phenylalanine deaminase test

Phenylalanine deaminase determines whether the organism possesses the enzyme phenylalanine deaminase that deaminates phenylalanine to phenylpyruvic acid, which reacts with ferric salts to give a green colour. Agar slants of the medium containing DL-phenylalanine is inoculated with a single colony of the test organism. After incubation at 37°C for 18–24 hours, 4 or 5 drops of 10% solution of ferric chloride reagent are added directly to the surface of the agar. If the test is positive, a green colour will develop in the fluid and in the slope. This test is useful in initial differentiation of *Proteus*, *Morganella*, and *Providencia* from the rest of the Enterobacteriaceae.

Decarboxylase tests

This test is based on the ability of some bacteria to decarboxylate an amino acid to the corresponding amine with the liberation of carbon dioxide. The production of decarboxylases is induced by a low pH and, as a result of their action, the pH rises to neutrality or above.

The test medium contains the Moeller decarboxylase base medium. This base contains peptone, meat extract, glucose, pyridoxal and two pH indicators, bromocresol purple and cresol red. The pH is adjusted to 6.0. The basal medium is divided into four portions and treated as follows:

1. Add 1% L-lysine hydrochloride.
2. Add 1% L-ornithine hydrochloride.
3. Add 1% L-arginine hydrochloride.
4. No addition (control).

Readjust pH to 6.0. Distribute 1 ml quantities in small tubes containing sterile liquid paraffin to provide a layer about 5 mm thick above the medium. Autoclave at 121°C for 15 minutes.

Inoculate lightly through the paraffin layer with a straight wire. Incubate at 37°C and read daily for 4 days. During the initial stages of incubation, both tubes turn yellow, owing to the fermentation of the small amount of glucose in the medium. If amino acid is decarboxylated, alkaline amines are formed and the medium reverts to its original purple colour. The role of glucose in the medium is important because decarboxylases are inducible enzymes produced in an acid pH. In addition, by definition all members of Enterobacteriaceae are glucose-positive, providing a growth stimulus.

Conversion of the control tube to a yellow colour indicates that the organism is viable and that the pH of the medium has been lowered sufficiently to activate the decarboxylase enzymes. Reversion of tube containing the amino acid to original purple colour indicates a positive test owing to the formation of amines from the decarboxylation reaction.

Bacteriocin, bacteriophage and serotyping

Each species of an organism contains a number of different strains. These epidemiological markers are useful for intraspecies differentiation of various strains.

Animal pathogenicity

Various experimental models used in diagnostic microbiology laboratory are mouse, rat, guinea pig, rabbit, nine-banded armadillo and monkey. Various routes of inoculation are intradermal, subcutaneous, intramuscular, intraperitoneal, intracerebral and intravenous. Oral and nasal routes can also be used. The identification of the organism is carried out on the basis of clinical and postmortem findings, and cultural characteristics.

Nucleic acid probes and polymerase chain reaction

See Chapter 7.

BACTERIAL TAXONOMY

The taxonomy of bacteria refers to three basic concepts—classification, nomenclature and identification.

Classification

It can be defined as the arrangement of organisms into taxonomic groups (taxa) on the basis of genotypic (genetic) and phenotypic (observable) similarities and differences. It allows the orderly grouping of microorganisms. For bacterial classification, three main approaches are usually followed. These include phylogenetic, Adansonian, genetic and intraspecies classification.

Phylogenetic classification

It is a hierarchical classification. It represents a branching tree-like arrangement, one characteristic being employed for division at each branch or level. This system is called phylogenetic because it denotes an evolutionary arrangement of species. Here some characteristics are given special weightage. For example, Gram staining, spore formation,

lactose fermentation, etc. are used to differentiate major groups, whereas less important properties such as nutritional requirements for growth of bacteria, production of certain enzymes by bacteria, etc. are employed to distinguish minor groups such as genera and species.

The formal levels of classification, in successive smaller subsets, are kingdom, phylum or division, class, order, family, tribe, genus and species. At present no standard classification of bacteria is universally accepted and applied although **Bergey's Manual of Systematic Bacteriology** is widely used as authoritative source.

Adansonian classification

Adansonian classification makes no phylogenetic assumption but merely takes into account all the characteristics expressed at the time of study. It gives equal weightage to all measurable features, and groups the organisms on the basis of similarities of several characteristics. The availability of computers has extended the scope of phenetic classification by permitting comparison of very large number of properties of several organisms at the same time. This is known as **numerical taxonomy**.

Genetic classification

This is based on homology of DNA base sequences of the microorganisms. DNA relatedness is determined by studying the nucleotide sequence of DNA by DNA hybridization or recombination methods. The study of messenger RNA and ribosomal RNA also provides useful information on genetic relatedness among bacteria. Genetic classification has been used more with viruses than with bacteria.

Intraspecies classification

Intraspecies classification is based on biochemical properties (biotypes), antigenic properties (serotypes), susceptibility to bacteriophages (phage types), resistance to various chemicals (resistotypes), or production of bacteriocins [colicin (klebocin and pyocin) types].

Nomenclature

It refers to the naming of microorganisms. It is governed by the *International Committee on Systematic Bacteriology* and published as *Approved List of Bacterial Names* in the *International Journal of Systematic Bacteriology*. By

accepted taxonomic convention, order names have the endings '-ales' (i.e. the order Eubacteriales), family names have the latinized ending '-aceae' (e.g. the family Enterobacteriaceae), and tribe names end in '-eae' (e.g. the tribe Proteae). The order, family and tribe names are capitalized. The genus name is also capitalized followed by species name which is not capitalized. If typed, the genus and species names should be italicized. If written, they should be underlined. Species should not be capitalized even when derived from the name of the person who discovered it. Often, the genus name is abbreviated by using the first letter of the genus followed by a period and the species epithet (name) (e.g. *E. coli*). The genus name followed by the word species (e.g. *Staphylococcus* species) may be used to refer to the genus as a whole. Species abbreviated sp. (singular) or spp. (plural) is used when the species is not specified. When the bacteria are referred to as a group, their names are neither capitalized nor underlined (e.g. staphylococci).

Identification

Suitable criteria for the purpose of identification include cell shape, Gram reaction and the presence or absence of specialized structures such as spores or flagella. Staining procedures such as Gram stain can provide reliable assessment of the nature of cell surfaces. Some bacteria produce characteristic pigments and others can be differentiated on the basis of their complement of extracellular enzymes (e.g. haemolysins). Tests such as oxidase test can be used to distinguish organisms on the basis of the presence of a respiratory enzyme, cytochrome C.

The traditional method of placing an organism into a particular genus and species is based on the similarity of all members in a number of phenotypic characteristics. This is accomplished by testing each bacterial culture for a variety of metabolic characteristics and comparing the results with those listed in established charts.

In rapid identification systems, a numerical taxonomy (also called computer taxonomy, phenetics, or taxometrics) is used. Numerical classification schemes use a large number (frequently 100 or more) of taxonomically useful characteristics. All these characteristics are assigned numerical value. The computer clusters different strains at selected levels of overall similarity (usually 80% at the species level) on the basis of the frequency with which they share traits.

KEY POINTS

- **Microorganisms** may be observed by *hanging drop preparation, dark-ground microscopy, Gram staining, Ziehl-Neelsen staining, Albert's staining, silver impregnation method, negative staining*, etc.
- Bacteria are divided into two major classes according to staining characteristics—*Gram-positive* (violet) and *Gram-negative* (red).
- Cell walls of some bacteria such as the *mycobacteria* contain lipids (mycolic acids); they are relatively impermeable to simple stains but when treated with hot concentrated carbolic fuchsin, subsequently resist decolorization with 20% sulphuric acid, these bacteria are called *acid-fast organisms*.
- Identity of the **microorganisms** can be confirmed by various *biochemical reactions*.
- The **taxonomy** of bacteria refers to three basic concepts; *classification, nomenclature* and *identification*.

- The traditional method of placing an organism into a particular **genus** and **species** is based on the similarities of all members in a number of **phenotypic characteristics**.
- **Species** may be subdivided into **subspecies**, **serovar** and **biovar**.
- For any one **species**, the **G + C content** is relatively **fixed**, or falls within a **very narrow range**.
- A **bacterial species** is a population of cells with similar characters.
- A **strain** is a subset of a bacterial species differing from other bacteria of the same species by some minor but identifiable difference.
- A **serovar** is a strain differentiated by serological means.
- A **biovar** is a strain that is differentiated by biochemical or other non-serological means.
- A **morphovar** is a strain which is differentiated on the basis of morphological means.
- An **isolate** is a pure culture derived from a heterogeneous, wild population of microorganisms.



Important Questions

1. Discuss methods of collection and transportation of specimens.
2. Enumerate criteria for rejection of specimens.
3. Write short notes on:

(a) Gram stain (c) Indole production (e) Voges-Proskauer test (g) Nitrate reduction test (i) Hydrogen sulphide production test (k) Oxidase test (m) Decarboxylase tests	(b) Ziehl-Neelsen stain (d) Methyl red test (f) Citrate utilization test (h) Urease test (j) Catalase test (l) Phenylalanine deaminase test
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4. Discuss in detail bacterial taxonomy.



Multiple Choice Questions

1. Which of the following organisms is **not** acid-fast?

(a) Tubercle bacilli. (b) Lepra bacilli. (c) <i>Nocardia</i> . (d) <i>Corynebacterium diphtheriae</i> .	(a) <i>Staphylococcus</i> . (c) <i>Proteus</i> .	(b) <i>Streptococcus</i> . (d) <i>Salmonella</i> .
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2. Silver impregnation method is used for demonstration of:

(a) <i>Treponema pallidum</i> . (c) <i>Shigella</i> .	(b) vibrios. (d) <i>Salmonella</i> .
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3. Urease test is **positive** in:

(a) <i>Klebsiella pneumoniae</i> . (b) <i>Escherichia coli</i> . (c) <i>Salmonella</i> serotype Typhi. (d) <i>Shigella flexneri</i> .	(a) <i>Proteus</i> . (b) <i>Morganella</i> . (c) <i>Providencia</i> . (d) <i>Escherichia</i> .
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4. Oxidase test is **negative** in:

(a) <i>Neisseria</i> . (c) <i>Vibrio</i> .	(b) <i>Pseudomonas</i> . (d) <i>Proteus</i> .
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5. Catalase production is **negative** in:

(a) <i>Staphylococcus</i> . (c) <i>Proteus</i> .	(b) <i>Streptococcus</i> . (d) <i>Salmonella</i> .
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6. Which of the following tests detects the production of acetoin?

(a) Methyl red test. (c) Indole test.	(b) Voges-Proskauer test. (d) Citrate test.
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7. Phenylalanine deaminase test is **negative** in:

(a) <i>Proteus</i> . (b) <i>Morganella</i> . (c) <i>Providencia</i> . (d) <i>Escherichia</i> .	(a) Gram staining. (b) Ziehl-Neelsen staining. (c) negative staining. (d) All of the above.
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8. Capsules are generally viewed by:



Answers

1. d 2. a 3. a 4. d 5. b 6. b 7. d 8. c

Sterilization, Disinfection and Biomedical Waste Management

Competency achievement: The student after reading the chapter should be able to:

MI1.4: Classify and describe the different methods of sterilization and disinfection. Discuss the application of the different methods in the laboratory, in clinical and surgical practice.

MI1.5: Choose the most appropriate method of sterilization and disinfection to be used in the laboratory, in clinical and surgical practice.

CM14.1: Define and classify hospital waste.

CM14.2: Describe various methods of treatment of hospital waste.

CM14.3: Describe laws related to hospital waste management.

SU14.1: Describe aseptic techniques, sterilization and disinfection.

SU15.1: Describe classification of hospital waste and appropriate methods of disposal.

Sterilization

It is defined as the process by which an article, a surface or a medium is freed of all microorganisms including viruses, bacteria, their spores and fungi, both pathogenic and non-pathogenic.

Disinfection

It is a process of destruction or removal of organisms capable of giving rise to infection. A **disinfectant** is a chemical or physical agent that is applied to inanimate objects to kill microbes. Disinfectants are capable of killing vegetative bacteria, fungi, viruses and rarely bacterial spores. Therefore, disinfection must never be used when sterilization is possible.

Antisepsis

It is the destruction or inhibition of microorganisms in living tissues thereby limiting or preventing the harmful effects of infection. A disinfectant that is applied to living tissue, to kill microbes, is referred to as an **antiseptic**. Note that not all disinfectants are antiseptics because an antiseptic additionally must not be so harsh that it damages living tissue.

Various agents used in sterilization and disinfection may be divided into:

A. Physical agents

1. Sunlight
2. Drying
3. Heat
4. Filtration
5. Radiations

B. Chemical agents

1. Phenols
2. Halogens
3. Metallic salts
4. Aldehydes
5. Alcohols
6. Dyes
7. Vapour-phase disinfectants
8. Surface active disinfectants

A. PHYSICAL AGENTS

1. SUNLIGHT

Sunlight possesses ultraviolet rays which along with heat rays are responsible for appreciable germicidal activity. These rays, however, cannot penetrate through glass, i.e. window panes. This is one of the natural methods of sterilization of water in tanks, rivers and lakes.

2. DRYING

Water constitutes 80% of the weight of the bacteria and is also essential for the growth of bacteria. Therefore, drying has deleterious effect on many bacteria. However, spores are unaffected by drying.

3. HEAT

Heat is the most reliable, certain and rapid method of sterilization. It can be easily controlled and unlike chemical disinfection, leaves no potentially harmful residue. Unless the material to be sterilized is heat-sensitive, this method should be preferred. The time required for sterilization by

heat is inversely proportional to the temperature of exposure, i.e. higher the temperature shorter the time of exposure.

Factors influencing sterilization by heat

- Number of microorganisms and spores present in the specimen.
- Species, strain and spore-forming ability of the microorganisms.
- Presence of organic material (e.g. serum, blood, etc.).
- Temperature.
- Contact time.

Types of heat and principle

There are two types of heat—dry heat and wet heat.

Dry heat

It is believed to kill microorganisms by causing destructive oxidation of essential cell constituents. Dry heat at 100°C for 60 minutes and 115°C for 60 minutes can kill all vegetative bacteria and fungal spores, respectively. Bacterial spores can be killed by dry heat at 160°C for one hour or 180°C for 20 minutes. On the whole, dry heat is less efficient sterilization process than moist heat.

Moist heat

It causes denaturation and coagulation of proteins. When steam condenses on cooler surface, it releases its latent heat and raises the temperature of its surface. If spores are present, steam condenses on them and increases their water content leading to hydrolysis and breakdown of bacterial proteins. Most vegetative bacteria are killed by moist heat at 50–65°C in 10 minutes. Resistance of bacterial spores varies with different strains of the same species. For example, spores of most strains of *Clostridium tetani* are killed by boiling at 100°C for 10 minutes. However, some strains resist boiling for 1–3 hours. The spores of some strains of *C. botulinum* resist boiling at 100°C up to 8 hours. However, all spores are killed by autoclaving at 121°C for 10–30 minutes.

I. Sterilization by dry heat

(A) Red heat

Inoculating wires and loops, points of forceps and spatulas are sterilized by holding them almost vertical in a Bunsen burner flame until red hot (Table 5.1).

(B) Flaming

Scalpel blades, needles, mouths of culture tubes and bottles are sterilized by passing the article through the Bunsen flame

without allowing them to become red hot. These items may be immersed in spirit in a tray and then burning off the spirit. However, it does not produce sufficient heat and destroys only vegetative organisms.

(C) Incineration

This is an efficient method for rapidly destroying contaminated materials such as soiled dressings, pathological materials, etc.

(D) Hot air oven

It is a method of choice for sterilization of glassware such as assembled all glass syringes, test tubes, Petri dishes, pipettes and flasks; metal instruments such as forceps, scissors and scalpels; sealed materials such as oils, jellies and powders which are impervious to steam; and swab sticks packed in test tubes. It is not suitable for materials like fabrics which may be damaged by heat.

Hot air oven is electrically heated and is fitted with a thermostat that maintains the chamber air at a chosen temperature and a fan that distributes hot air in the chamber (Fig. 5.1). It must not be overloaded and spaces must be left for circulation of air through the load. Holding time for sterilization in hot air oven is **1 hour at 160°C** or **20 minutes at 180°C**. It is timed as beginning when the thermometer first shows 160°C or 180°C, respectively. Sterilization controls by hot air oven are given in Box 5.1.

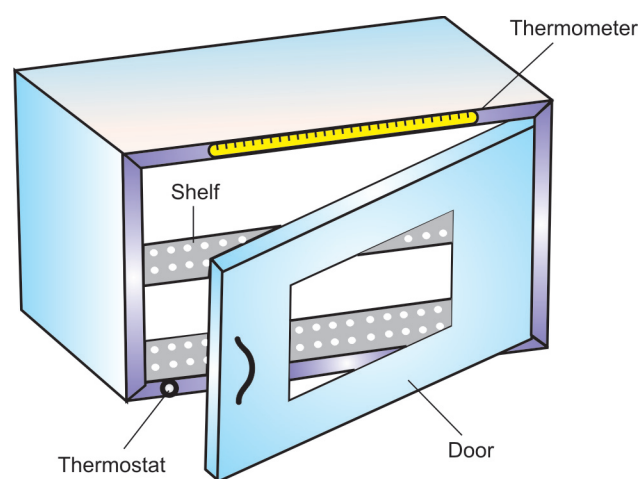


Fig. 5.1: Hot air oven.

Table 5.1: Sterilization by dry heat

Mode of sterilization	Instrument	Temperature and time	Sterilization of	Advantages/disadvantages
Red heat	Bunsen burner	Till red hot	Inoculating wire loops, forceps and spatulas	Sterilization is rapid and thorough
Flaming	Bunsen burner	Waving through the flame	Scalpel blades, glass slides, mouths of culture tubes and bottles	1. Surface sterilization is possible 2. Rapid method
Hot air	Hot air oven	160°C for 1 hour or 180°C for 20 minutes	Glassware, sealed materials like oils, greases, dry powder, etc.	Can be used for loads that cannot be penetrated by steam

Box 5.1: Sterilization by hot air oven**Sterilization controls**

Two types of controls are available:

1. Biological control

An envelope containing a filter paper strip impregnated with 10^6 spores of *Bacillus subtilis* subsp. *niger* (NCTC 10075 or ATCC 9372) is placed within the load in hot air oven (Table 5.2). After sterilization is over, the strip is removed and inoculated into tryptone soy broth and incubated anaerobically at 37°C for 5 days. No growth of *B. subtilis* subsp. *niger* indicates proper sterilization.

2. Chemical control

A Browne's tube containing red solution is placed within the load. A change of colour of the solution from **red** to **green** indicates proper sterilization.

Table 5.2: Biological controls of different sterilization methods

Method of sterilization	Biological control
Hot air oven	<i>Bacillus subtilis</i> subsp. <i>niger</i>
Autoclave	<i>Bacillus stearothermophilus</i>
Low temperature steam-formaldehyde	<i>Bacillus stearothermophilus</i>
Ethylene oxide	<i>Bacillus globigi</i> (a red-pigmented variant of <i>Bacillus subtilis</i>)
Ionizing radiations	<i>Bacillus pumilis</i>
Filtration	<i>Serratia marcescens</i> , <i>Pseudomonas diminuta</i>

II. Sterilization by moist heat

Sterilization by moist heat means killing of the micro-organisms with hot water or steam. Moist heat is divided into three forms (Table 5.3):

- A. At temperatures below 100°C.
- B. At a temperature of 100°C.
 - (a) Boiling water
 - (b) Free steam
- C. At temperature above 100°C.

(A) Moist heat at temperatures below 100°C

Heat-labile fluids may be disinfected (not sterilized) by heating at temperatures below 100°C. Such treatment is sufficient to kill mesophilic vegetative bacteria. This includes:

- **Pasteurization** of milk. The temperature employed is either **63°C for 30 minutes** (holder method) or **72°C for 20 seconds** (flash method) followed by rapid cooling to 13°C or lower. By this method non-sporing organisms such as mycobacteria, *Brucella* and salmonellae are destroyed. However, *Coxiella burnetii*, causative agent of Q fever, may survive pasteurization of milk by holder method and is at borderline of inactivation by the flash method.

The flash method is preferable for pasteurization of milk because it is less likely to change the flavour and nutrient content, and it is more effective against resistant pathogens such as *C. burnetii*. Although pasteurization inactivates most viruses and vegetative stages of 97–99% of bacteria and fungi, they do not kill spores and thermoresistant species (mostly non-pathogenic lactobacilli, micrococci and yeasts). Therefore, milk is not sterile after regular pasteurization, which explains why even an unopened carton of milk will eventually spoil on prolonged storage.

- **Heat-labile fluids** such as serum may be disinfected by heating at 56°C for 1 hour. If temperature rises above 59°C it will coagulate.
- **Vaccines** prepared from non-sporing bacteria may be inactivated in a water bath at 60°C for 1 hour.
- **Household utensils** and **patient's clothing** may be disinfected by washing in water at 70–80°C for several minutes.
- Media such as Lowenstein-Jensen and Loeffler's serum slope are rendered sterile by heating at 80–85°C for half an hour on three successive days in an inspissator.
- Items which cannot withstand heat at 100°C may be disinfected by steam at sub-atmospheric pressure at a temperature of 75°C with formaldehyde vapour. This is known as **low temperature steam-formaldehyde (LTSF) sterilization**.

Bacillus stearothermophilus (NCTC 10003 or ATCC 7953) has been used to test the efficacy of LTSF sterilizers (Table 5.2).

Table 5.3: Sterilization by moist heat

Mode of sterilization	Instrument	Temperature and time	Sterilization of	Advantages/disadvantages
Below 100°C	Water bath	56°C for 1 hour	Serum	May be used for disinfection. Most vegetative mesophilic bacteria are killed
At 100°C	Boiling water bath	100°C for 10–20 min.	Glass, metal and rubber items	Kills all vegetative bacteria and some spores
Steaming at 100°C	Arnold steamer	100°C for 20 min. on 3 successive days	Culture media containing sugar and gelatin	Prevents decomposition of media. <i>Spores of thermophilic bacteria may escape killing</i>
Steaming above 100°C	Autoclave	121°C for 15–20 min.	Culture media and other aqueous solutions, dressing material, linen, gloves, etc.	Most reliable method of sterilization

(B) Moist heat at a temperature of 100°C

(a) **Boiling at 100°C:** Boiling at 100°C for 10–30 minutes kills all vegetative bacteria and some bacterial spores. Therefore, it is not recommended for sterilization of instruments for surgical procedures.

(b) **Free steam at 100°C:** Steam at normal atmospheric pressure is at 100°C. But, in addition, it has latent heat which on condensing on the article to be sterilized releases its latent heat. A **Koch or Arnold steam sterilizer** consists of a vertical metal cylinder with a removable conical lid having a small opening for the escaping steam. Water is added on the bottom and there is a perforated shelf above water level (Fig. 5.2). On this shelf articles to be sterilized are placed.

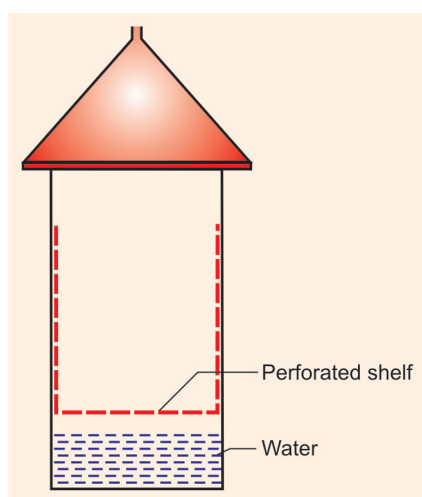


Fig. 5.2: Steam sterilizer.

One single exposure to steam for 90 minutes ensures complete sterilization but for media containing sugar and gelatin, which may get decomposed on long heating, an exposure of **100°C for 20 minutes on three consecutive days** is employed. This is known as **tyndallization or intermittent sterilization**. First exposure to steam kills all vegetative bacteria, and any spores present being in a favourable medium, will germinate and will be killed on the subsequent occasions. Therefore, non nutrient media cannot be sterilized by this method.

(C) Moist heat at temperature above 100°C

Steam above 100°C or saturated steam is a more efficient sterilizing agent than hot air because:

- It provides greater lethal action of moist heat,
- It is quicker in heating up the exposed articles, and
- It can penetrate easily porous material such as cotton wool stoppers, paper and cloth wrappers, bundles of surgical linen, and hollow apparatus.

When the steam meets the cooler surface of the article, it condenses into a small volume of water and liberates its considerable latent heat to that surface, for example, 1600 ml of steam at 100°C, i.e. at atmospheric pressure condenses into 1 ml of water at 100°C liberating 518 calories of heat. The large contraction in volume brings more steam to the

same site and the process continues until the temperature of the article is raised to that of steam. The water of condensation ensures moist conditions for killing of the exposed micro-organisms. Pure steam must be used and the presence of air avoided since air hinders penetration by the steam.

Sterilization by **steam under pressure (autoclaving)** is suitable for culture media and aqueous solutions (since atmosphere of steam prevents evaporation during heating), dressing material, linen, gloves, etc. Satisfactory sterilization can be achieved at 15 pounds per square inch (psi) pressure equivalent to **121°C in 15–20 minutes**. In fact, the only practical and dependable method of sterilization is steam under pressure using different types of autoclaves. *However, the autoclave is ineffective for sterilizing substances that repel moisture (oils, waxes or powders).*

All the air must be removed from the autoclave chamber and the articles to be sterilized so that the latter are exposed to pure steam. There are three reasons for this:

- The admixture of air with steam results in lower temperature being achieved.
- Air hinders penetration of steam into the interstices of porous materials, surgical dressings, syringes, etc.
- The air being denser forms a separate and cooler layer in the lower part of the autoclave, so it prevents adequate heating of articles there.

Several types of steam sterilizers are available. The **laboratory autoclave or pressure cooker type autoclave** (Fig. 5.3) consists of a vertical or horizontal cylinder of gun metal or stainless steel in a supporting frame or case. The lid is fastened by screw clamps and rendered airtight by asbestos gasket. Lid bears a steam release valve or safety valve. It opens and closes when the steam pressure rises or falls the desired level, respectively. On its upper part of the side, the autoclave has a discharge tap for air and steam, and an air and steam release knob. Heating is done by electricity. Water

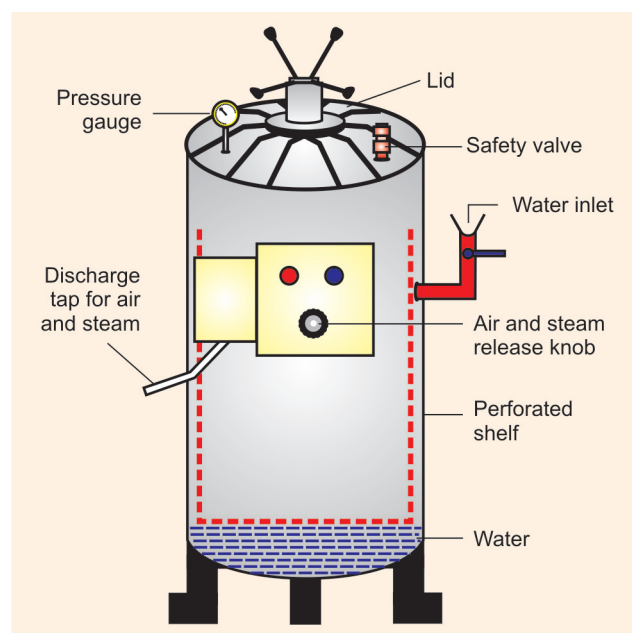


Fig. 5.3: Autoclave.

is added on the bottom of the autoclave. Above this is a perforated shelf on which articles to be sterilized are placed.

The lid is closed, discharge tap is opened and safety valve is adjusted to the required pressure. As heating continues, the steam and air mixture escapes. To know when all the air inside the autoclave has escaped the discharge tap is connected with one end of a rubber tube and the other end of it is placed in water. When the air bubbles stop coming it indicates that all the air from inside the autoclave has been removed. The discharge tap is now closed. Steam pressure rises inside and when it reaches the desired set level (15 psi) the safety valve opens and excess steam escapes. From this point the **holding time** (15 minutes) is counted.

When the holding time is over, the heating is stopped and autoclave allowed to cool till pressure gauge indicates that inside pressure has reached to the atmospheric pressure. The discharge tap is now opened and air is allowed to enter the autoclave. The lid is now opened and the sterilized articles removed. If the tap or lid is opened when the pressure inside is high, the liquid media boil violently and may explode. On the other hand, if the articles are not removed for a long time after the normal atmospheric pressure has reached inside the autoclave, an excessive amount of water will be evaporated and lost from the media. Sterilization controls by autoclaving are given in Box 5.2.

Box 5.2: Sterilization by autoclaving

Sterilization controls

Two types of controls are available:

1. Biological control

An envelope containing a filter paper strip impregnated with 10^6 spores of *Bacillus stearothermophilus* (NCTC 10003 or ATCC 7953) is placed with the load in the coolest and least accessible part of the autoclave chamber (Table 5.2). After sterilization is over the strip is removed and inoculated into tryptone soy broth and incubated at 56°C for 5 days. No growth of *B. stearothermophilus* indicates proper sterilization. Spores of this organism withstand 121°C for up to 12 minutes and this has made the organism ideal for testing autoclaves.

2. Chemical control

A Browne's tube containing red solution is placed within the load. A change of colour of the solution from **red to green** indicates proper sterilization.

4. FILTRATION

Filtration method may be used with both liquids and air. Liquids such as sera and solutions of heat-labile substances such as sugars and urea, used for preparation of media, can be sterilized by filtration. Filtration does not kill microbes, it separates them out. This method is also useful for:

- Sterilization of pharmaceutical substances,

- Separation of bacteriophages and bacterial toxins from bacteria, and
- Isolation of organisms which are scanty in fluids.

The filter disc retains the organisms which can then be cultured.

Limitations

Mycoplasma and viruses, which are very small, cannot be kept back by the bacterial filters, therefore, serum sterilized by filtration cannot be employed for clinical use. *Serratia marcescens* and *Pseudomonas diminuta* have been used to test the efficacy of different filters (Table 5.2).

Types of filters

1. Earthenware filters

Important examples are earthenware filters made from kieselguhr (a fossil diatomaceous earth), and chamberland filters made of unglazed porcelain. They are made in the form of hollow candles with different grades of porosity. The fluid to be sterilized is passed through the candle. After use they can be sterilized by scrubbing with stiff brush followed by boiling and autoclaving.

2. Asbestos (Seitz) filters

They are made up of a disc of asbestos (magnesium trisilicate). It is supported on a perforated metal disc within a metal funnel. The latter with filter disc fitted is sterilized by autoclaving. It is then fitted onto a sterile flask through a silicone rubber bung. The fluid to be sterilized is put into the funnel and flask connected to the exhaust pump through its side tap. Sterilized fluid is collected from the flask and filter disc is discarded after use. These discs are available with different grades of porosity.

3. Sintered glass filters

These are made of finely ground glass fused together. These are available in different pore sizes. They are also available in the form of a disc fused into a glass funnel. After use they are washed with running tap water in reverse direction and cleaned with warm, strong sulphuric acid.

4. Membrane filters

These are made up of cellulose esters and are widely used nowadays for sterilization of heat-labile fluids. These are also known as millipore filters. They are available as discs of pore size varying from 0.015–12 μm . The 0.22 μm filter is the most commonly used because the pore size is smaller than that of bacteria. They can also be used for bacterial counts of water. A known amount of water is filtered through the membrane filter disc. The upper side of the disc is then placed on an appropriate moist culture medium and incubated. The colonies that develop can be counted and viable count calculated.

5. Syringe filters

Syringes fitted with membranes of 13 mm and 25 mm diameter are available. The fluid to be sterilized is forced through the disc by pressing the piston of the syringe.

6. Air filters

Filtration of air is accomplished with the use of high-efficiency particulate air (HEPA) filters. HEPA filters are able to remove microorganisms larger than 0.3 μm and are used in laboratory hoods, operation theatres, and in rooms of immunocompromised individuals.

5. RADIATIONS

Two types of radiations are used:

- Non-ionizing.
- Ionizing.

Non-ionizing radiations

These include infrared and ultraviolet radiations:

Infrared rays

Infrared rays bring about sterilization by generation of heat. Articles to be sterilized are placed in a moving conveyor belt and passed through a tunnel that is heated by infrared radiators to a temperature of 180°C. The articles are exposed to that temperature for a period of 7.5 minutes. Articles sterilized include metallic instruments and glassware. It is mainly used in central sterile supply department. Efficiency can be checked by using Browne's tube No. 4 (blue spot).

Ultraviolet (UV) radiations

Ultraviolet (UV) radiations in the range of 250–260 nm wavelength are highly effective. Low pressure mercury vapour lamps emit over 95% of radiations with the wavelength of 253.7 nm. Most vegetative bacteria are susceptible to UV radiations. Spores are highly resistant and susceptibility of viruses is variable. **Human immunodeficiency virus is not inactivated by UV radiations.** UV radiations interfere with DNA replication. They can penetrate only a few mm into liquids and not at all into solids. Therefore, their use is best restricted to disinfection of clean surfaces like *inoculation hoods, laboratories, wards, and operation theatres*. Source of UV radiations must be shielded to prevent the radiations falling on eyes and skin because it may damage them.

Ionizing radiations

These include *X-rays, gamma rays* and *cosmic rays*. These have very high penetrative power and are highly lethal to all cells including bacteria. They damage DNA by various mechanisms. Spores are more resistant than vegetative bacteria. Large commercial plants use gamma radiations from a cobalt 60 source for sterilization of prepacked disposable items such as *plastic syringes, transfusion sets, catheters, cannulas, culture plates*, etc. that are unable to withstand heat because there is no appreciable increase in the temperature. Therefore, this method is known as **cold sterilization**. High cost of installation limits its use commercially. *Bacillus pumilis* has been used to test the efficacy of ionizing radiations (Table 5.2).

B. CHEMICAL AGENTS

Several chemical agents are used as antiseptics and disinfectants. An ideal antiseptic or disinfectant should:

- Have a wide spectrum of activity and must be effective against all microorganisms.
- Be active in the presence of organic matter.
- Be effective in both acid and alkaline media.
- Have penetrating power.
- Be stable.
- Not corrode metals.
- Not cause local irritation or sensitization.
- Not be toxic if absorbed into circulation.
- Not interfere with healing.
- Be cheap and easily available.

Following factors influence the potency of disinfectants.

- Concentration.
- Time of action.
- pH of medium.
- Temperature.
- Nature of organisms.
- Presence of organic matter.

1. PHENOLS

Lister, the father of antiseptic surgery, introduced the use of phenol (carbolic acid) in surgery. Phenols are obtained by distillation of coal tar and have a powerful microbicidal action. They cause cell membrane damage thus releasing cell contents and causing cell lysis. They are resistant to inactivation by organic matter and are active against Gram-positive and Gram-negative bacteria, moderately active against mycobacteria, and have little activity against spores and viruses. They are used mainly for *discarded cultures, contaminated pipettes and other infected material*. Phenol is bactericidal at a concentration of 1%. At a concentration of 0.5% it is used for preservation of sera and vaccines.

Phenol derivatives

Certain phenol derivatives like cresols, chlorhexidine, chloroxylenol and hexachlorophene are commonly used as disinfectants:

- **Cresols:** Cresols are obtained by distillation of coal tar, are emulsified with green soap and sold under the trade names of *Lysol* and *Creolin*. They are active against a wide range of organisms. They are most commonly used for sterilization of infected glasswares, cleaning floors and disinfection of excreta. They are not inactivated by the presence of organic matter.
- **Chlorhexidine:** It is a relatively non-toxic skin disinfectant. It is most active against Gram-positive organisms, and fairly effective against Gram-negative ones. Aqueous solutions are used in the treatment of wounds.
- **Chloroxylenol (Dettol):** It is less toxic and less irritant, and is also less active. It is readily inactivated by organic matter. It is inactive against *Pseudomonas*.

- **Hexachlorophene:** It is more active against Gram-positive than Gram-negative bacteria. It is applied on skin for prophylaxis against staphylococcal infections. It is bacteriostatic at very high dilutions. It is potentially toxic and should be used with care.

2. HALOGENS

Halogens are oxidizing agents. They cause damage by oxidation of essential sulfhydryl groups of enzymes. Chlorine and iodine are the halogens which are used as disinfectants. They are bactericidal and sporicidal. They are active in very high dilutions and their action is very rapid. In addition to chlorine itself there are three types of chlorine compounds, the hypochlorites, and the inorganic and organic chloramines. **The disinfectant action of all the chlorine compounds is due to release of free chlorine.** When elemental chlorine or hypochlorites are added to water, the chlorine reacts with water to form hypochlorous acid. It is a strong oxidising agent and effective disinfectant. The activity of chlorine is markedly influenced by the presence of organic matter. Sodium hypochlorite, chloramine and bleaching powder are most widely used for disinfection of HIV infected material. Hypochlorite solution decays rapidly, therefore, it should be prepared daily.

- **Chlorine** has a special place in the treatment of water supply, and combinations of hypochlorite and detergents are useful for cleansing and disinfection in food and dairy industry. Hypochlorites have a wide spectrum of activity against viruses and very little activity against tubercle bacilli. They are available in liquid or powder form as salts of calcium, lithium and sodium.
- **Iodine** in alcoholic and aqueous solutions is used almost exclusively as a skin disinfectant (antiseptic). Like chlorine, it is also inactivated by organic matter. It is also active against tubercle bacilli and a number of viruses. Mixtures of iodine with various surface active agents that act as carriers for iodine are known as **iodophores**, an example of this is **betadine**. It can be used as a **bactericidal antiseptic for intact skin** and for disinfection of superficial wounds. It is also active against fungi and *Trichomonas*.

3. METALLIC SALTS

All metallic salts have some degree of toxicity for bacteria. The most toxic are those of mercury and silver and the least toxic are those of sodium and potassium. Mercury compounds act on bacteria by combining with sulfhydryl (SH) groups of bacterial proteins and other essential intracellular compounds. Merthiolate (sodium ethylmercurithiosalicylate) is used in a dilution of 1 in 10,000 for preservation of sera. In the past, 1% solution of silver nitrate was instilled in the eye in newborn babies for the prophylaxis of gonococcal ophthalmia neonatorum.

4. ALDEHYDES

Two aldehydes (formaldehyde and glutaraldehyde) are currently of considerable importance, although others also possess antimicrobial activity. They act through alkylation of amino, carboxyl or hydroxyl groups, and probably damage nucleic acids.

Formaldehyde

Formaldehyde is an aldehyde generally used as formalin, a 37% aqueous solution of formaldehyde gas. Formaldehyde gas is often used for sterilization of rooms, furniture and a wide variety of articles likely to be damaged by heat, such as clothing, woollen blankets, mattresses, respirators, heat-sensitive instruments, etc. However, its usefulness is limited by its irritability factor and its potential carcinogenicity. Therefore, formaldehyde should not be used as a disinfectant or sterilant on a routine basis.

Glutaraldehyde

It is more effective and less irritant than formaldehyde. It possesses high microbicidal activity against bacteria and their spores, mycelial and spore forms of fungi, and various types of viruses, including human immunodeficiency viruses and enteroviruses. 2% alkaline buffered solution is germicidal in approximately 10 minutes and sporicidal in 3–10 hours. It is used for sterilization of heat-sensitive instruments like *cystoscopes*, *bronchoscopes*, *thermometers*, etc. *Glutaraldehyde is toxic and irritant to skin and mucous membranes, therefore, contact with it must be avoided. It must be used in a fume-hood or in well ventilated area.*

5. ALCOHOLS

Several alcohols possess antimicrobial activity. The antimicrobial activity of alcohols can be attributed to their ability to denature proteins. Alcohol solutions containing 60–70% alcohol are most effective, and higher concentrations are less potent because proteins are not denatured easily in the absence of water. They are active against vegetative bacteria including tubercle bacilli, fungi and lipid-containing viruses but not against spores.

- **Isopropyl alcohol** is preferred over ethyl alcohol as it is a better fat solvent, more bactericidal and less volatile. It is commonly used for *disinfection of clinical thermometers*.
- **Methyl alcohol** is effective against fungal spores.
- **Alcohol-based hand rubs** are recommended for the decontamination of highly soiled hands in situations where proper hand-washing is inconvenient or not possible.

6. DYES

Two groups of dyes—the aniline dyes and the acridines dyes have been used extensively as skin and wound antiseptics. Both groups are bacteriostatic in high dilution but have low bactericidal activity. They are much more active against Gram-positive than Gram-negative bacteria.

- **Aniline dyes** include brilliant green, malachite green and crystal violet. Their activity is inhibited by the presence of organic matter like pus. **They have no activity against tubercle bacilli, therefore, addition of malachite green to Lowenstein-Jensen medium makes it selective for the isolation of tubercle bacilli.** They interfere with the synthesis of the peptidoglycan component of the cell wall.
- **Acridine dyes** include acriflavine, proflavine, euflavine and aminacrine. They are little, if at all, affected by the presence of organic matter. Acridine dyes interfere with the synthesis of nucleic acids and proteins in both bacterial and mammalian cells.

7. VAPOUR-PHASE DISINFECTANTS

Two most important vapour-phase agents are ethylene oxide and formaldehyde.

Ethylene oxide (ETO)

It is a colourless gas soluble in water. It is highly lethal to all kinds of microbes including spores and tubercle bacilli. It is useful for sterilization of articles liable to be damaged by heat, e.g. *plastic and rubber articles, blankets, pharmaceutical products (crude drugs and powders) and complex apparatus such as heart-lung machines.*

It is highly effective sterilizing agent because it rapidly penetrates packing materials, even plastic wraps. It forms explosive mixture when more than 3% is present in air. This hazard can be overcome by using a mixture of 10% ethylene oxide in carbon dioxide. **It has an alkylating action on proteins.** Inhibition produced by it is irreversible, resulting in enzyme modification and inhibition of enzyme activity. *Bacillus globigi*, a red-pigmented variant of *B. subtilis*, has been used to test ethylene oxide sterilizers (Table 5.2).

Formaldehyde gas

It is liberated by spraying or heating of formalin, by addition of formalin to potassium permanganate or by volatilization of paraformaldehyde. Its antimicrobial activity depends upon several factors. The atmosphere must have a high relative humidity (more than 60% and preferably 80 to 90%) and a temperature of at least 18°C.

It is used for fumigation of operation theatres, wards and laboratories. After sealing the windows and other outlets, formaldehyde gas is generated by adding 150 g of potassium permanganate to 280 ml formalin for every 1000 cubic feet of room volume. The rooms are left unopened for 48 hours. Then open the doors and windows to allow vapours to disperse and neutralize any residual formaldehyde with ammonia by exposing 250 ml of SG 880 ammonia per litre of formalin used.

Beta-propiolactone (BPL) is a condensation product of ketone and formaldehyde. **It is an alkylating agent that acts through alkylation of carboxyl and hydroxyl groups.** It is active against all microorganisms including viruses. In

the liquid form it is used to sterilize vaccines and sera. It is more efficient than formaldehyde for fumigation purpose. BPL destroys microorganisms more readily than ethylene oxide but does not penetrate materials well and **may be carcinogenic. For these reasons, BPL has not been used as extensively as ethylene oxide.**

Hydrogen peroxide fogging

This is a better method replacing fumigation. It is done by fogging machine using hydrogen peroxide as disinfectant. It has the advantage of short cycle time and is non-toxic.

8. SURFACE ACTIVE DISINFECTANTS

Substances that alter the energy relationships at interfaces leading to reduction of surface or interfacial tension, are known as **surface active agents** or **surfactants**. They possess both hydrophobic (water-repelling) and hydrophilic (water-attracting) groups. On the basis of charge or the absence of ionization of the hydrophilic group, these surfactants are classified into anionic, cationic, non-ionic and amphoteric compounds.

Cationic compounds such as quarternary ammonium compounds are the most important surfactants. **These act on phosphate groups of cell membrane phospholipids and also enter the cell.** This leads to loss of membrane semi-permeability and leakage from the cell of nitrogen and phosphorus containing compounds. The agent which enters the cell, denatures its proteins. They possess strong bactericidal but weak detergent properties. They are essentially bacteriostatic and are more active against Gram-positive than Gram-negative bacteria. *Pseudomonas aeruginosa* is particularly resistant to these agents.

They are also fungistatic and active against viruses with lipid envelopes (e.g. herpes and influenza) and much less against non-enveloped viruses (e.g. enteroviruses). Commercially available preparations of cationic compounds include cetrimide (cetavalon), benzalkonium chloride and laurodin. These compounds are most active in alkaline pH. Antimicrobial activity is affected greatly by acidic pH, organic matter and anionic surface active agents such as ordinary soaps.

Anionic surfactants such as common soaps usually have strong detergent but weak antimicrobial properties. These agents are most active at acid pH and effective against Gram-positive organisms but are relatively ineffective against Gram-negative organisms. These compounds cause gross disruption of lipoprotein framework of the cell membrane.

Amphoteric agents such as tego compounds possess detergent properties of anionic and antimicrobial activity of cationic compounds. They are active over a wide range of pH but their activity is markedly reduced by organic matter. At a concentration of 1% in water they are effective against a wide range of Gram-positive and Gram-negative organisms and some viruses.

Table 5.4 gives a list and the recommended concentrations of disinfectants commonly used in the hospitals.

Table 5.4: List and the recommended concentrations of disinfectants commonly used in the hospitals

Disinfectant	Recommended concentration
Ethanol	70%
Methylated spirit	70%
Glutaraldehyde	2% activated (available commercially as Cidex)
Bleaching powder (calcium hypochlorite)	14 g/litre of water
Sodium hypochlorite	1%, 0.1%
Hydrogen peroxide	3%
Lysol	2.5%
Savlon®	2.0%, 5.0%
Dettol®	4.0%
Betadine	2.0%

Testing of disinfectants

The tests most commonly used for testing of disinfectants are as follows.

1. Minimum inhibitory concentration (MIC)

This test measures the lowest concentration of the disinfectant that inhibits the growth of *Salmonella* serotype Typhi in a nutrient medium.

2. Phenol coefficient test

In this test similar quantities of organisms are added to rising dilutions of phenol and of the disinfectant to be tested. In the UK the organisms used are *S. serotype Typhi* and in the USA, *S. serotype Typhi*, *Staphylococcus aureus* and *P. aeruginosa* are used. For testing, two methods, i.e. **Rideal-Walker** and **Chick-Martin** can be used. In the latter, but not the former, a source of organic matter (e.g. 2% killed yeast suspension or 20% inactivated horse serum or 3% dried human faeces) is used to simulate natural situations. The dilution of the disinfectant in question which kills the organisms in a given time is divided by the dilution of phenol which kills the organisms at the same time. This gives the phenol coefficient. A phenol coefficient of 1.0 means that the disinfectant in question has the same effectiveness as phenol; a coefficient of less than 1.0 means it is less effective and more than 1.0 means it is more effective.

3. Capacity test (Kelsey and Sykes test)

Capacity test is designed to simulate the natural conditions under which the disinfectants are used in hospitals. The main feature of the test is that instead of one addition of a large inoculum of the test organism, the additions are made in increments with or without organic matter. This gives a

measure of the capacity of the disinfectant to cope with successive bacterial invasions. Test organisms (*S. aureus*, *Escherichia coli*, *P. aeruginosa* and *Proteus vulgaris*) in both clean (test bacteria in broth) and dirty (bacteria in 20% inactivated horse serum or 2% yeast suspension) conditions are added to the disinfectant in 3 successive lots at 0, 10 and 20 minutes. Each addition is in contact with the disinfectant for 8 minutes, therefore, samples are transferred at 8, 18 and 28 minutes, respectively to a recovery medium. The disinfectant is judged on its overall performance, i.e. its ability to kill bacteria as judged by recovery on subculture and not by comparison with phenol.

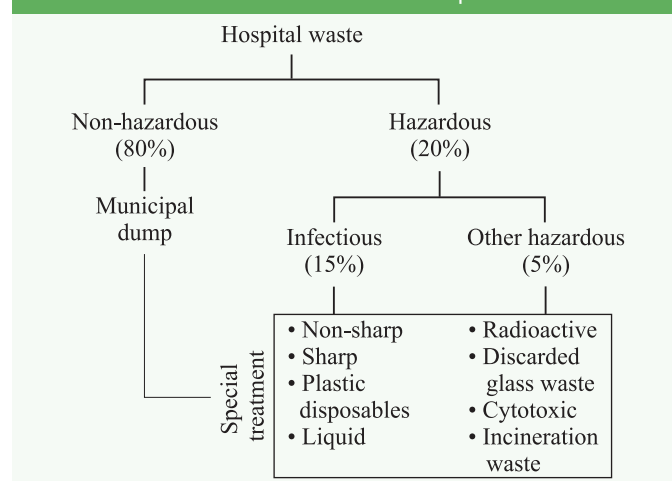
BIOMEDICAL WASTE MANAGEMENT RULES

The Ministry of Environment and Forests formulated biomedical waste rules in 1998. Subsequently, new guideline was published in 2016 with an amendment added in 2018 and 2019 Hospitals generate various kinds of wastes from wards, operation theatres and outpatient areas. These wastes include bandages, cotton, soiled linen, body parts, sharps (needles, syringes, etc.), medicines (discarded or expired), laboratory wastes, etc. Wastes which carry infective organisms should be properly collected, segregated, stored, transported, treated and disposed to prevent nosocomial infection. According to the Ministry of Environment and Forests gross generation of biomedical waste (BMW) in India is 4,05,702 kg/day of which only 2,91,983 kg/day is disposed, which means that almost 28% of the wastes is left untreated and not disposed finding its way in dumps or water bodies and re-enters our system.

Biomedical waste (BMW)

It is a broader term applied to waste generated in the diagnosis, treatment or immunization of human beings or animals, in research or in the production or testing of biological products. It also includes waste coming out of medical treatment given at home.

Table 5.5: Classification of hospital waste



- **Infectious wastes** include all those medical wastes, which have the potential to transmit viral, bacterial or parasitic diseases. It includes both human and animal infectious waste and waste generated in laboratories, and veterinary practice. Infectious waste is hazardous in nature.
- **Hazardous waste** is a waste which has a potential to pose a threat to human health and life. The persons most at risk are the staff of hospitals particularly nurses and waste handlers. In countries such as India, scavengers and rag-pickers are at serious risk.

It is important to note that not all hospital waste has the potential to transmit infection. It is estimated that 80% is

non-infectious general waste, 15% is infectious and 5% other hazardous waste (Table 5.5). However, if the infectious component gets mixed with the general non-infectious waste, the entire bulk of waste becomes potentially infectious.

Waste segregation

Waste should be segregated at source in bags of different colours to facilitate appropriate treatment and disposal. Table 5.6 shows biomedical waste categories and their segregation, collection, treatment, processing and disposal options.

Table 5.6: Biomedical waste categories and their segregation, collection, treatment, processing and disposal options

Category	Type of waste	Type of bag or container to be used	Treatment, processing and disposal options
Yellow	(a) Human anatomical waste: Human tissues, organs, body parts and foetus below the viability period (as per the Medical Termination of Pregnancy Act 1971, amended from time to time). (b) Animal anatomical waste: Experimental animal carcasses, body parts, organs, tissues, including the waste generated from animals used in experiments or testing in veterinary hospitals or colleges or animal houses. (c) Soiled waste: Items contaminated with blood, body fluids like dressings, plaster casts, cotton swabs and bags containing residual or discarded blood and blood components. (d) Expired or discarded medicines: Pharmaceutical waste like antibiotics, cytotoxic drugs including all items contaminated with cytotoxic drugs along with glass or plastic ampoules, vials, etc. (e) Chemical waste: Chemicals used in production of biologicals and used or discarded disinfectants. (f) Chemical liquid waste: Liquid waste generated due to use of chemicals in production of biologicals and used or discarded disinfectants, silver X-ray film developing liquid, discarded formalin, infected secretions, aspirated body fluids, liquid from laboratories and floor washings, cleaning, housekeeping and disinfecting activities, etc.		Incineration or plasma pyrolysis or deep burial*. Incineration or plasma pyrolysis or deep burial*. In the absence of above facilities, autoclaving or microwaving/hydroclaving followed by shredding or mutilation or combination of sterilization and shredding. Treated waste to be sent for energy recovery. Expired cytotoxic drugs and items contaminated with cytotoxic drugs to be returned to the manufacturer or supplier for incineration at temperature >1200°C or to common biomedical waste treatment facility or hazardous waste treatment, storage and disposal facility for incineration at >1200°C or encapsulation or plasma pyrolysis at >1200°C. All other discarded medicines shall be either sent back to manufacturer or disposed by incineration. Disposed of by incineration or plasma pyrolysis or encapsulation in hazardous waste treatment, storage and disposal facility. After resource recovery, the chemical liquid waste shall be pre-treated before mixing with other waste water.

(Contd...)

Table 5.6: Biomedical waste categories and their segregation, collection, treatment, processing and disposal options (*Contd...*)

Category	Type of waste	Type of bag or container to be used	Treatment, processing and disposal options
	(g) Discarded linen, mattresses, beddings contaminated with blood or body fluid.	Non-chlorinated yellow plastic bags or suitable packing material	Non-chlorinated chemical disinfection followed by incineration or plasma pyrolysis or for energy recovery. In the absence of above facilities, shredding or mutilation or combination of sterilization and shredding. Treated waste to be sent for energy recovery or incineration or plasma pyrolysis.
	(h) Microbiology, biotechnology and other clinical laboratory waste: Blood bags, laboratory cultures, stocks or specimens of microorganisms, live or attenuated vaccines, human and animal cell cultures used in research, industrial laboratories, production of biologicals, residual toxins, dishes and devices used for cultures.	Autoclave-safe plastic bags or containers	Pre-treat to sterilize with non-chlorinated chemicals on-site as per National AIDS Control Organization or World Health Organization guidelines thereafter for incineration.
Red	Contaminated waste (recyclable): Wastes generated from disposable items such as tubings, bottles, intravenous tubes and sets, catheters, urine bags, syringes (without needles and fixed needle syringes) and vacutainers with their needles cut) and gloves.	Red-coloured non-chlorinated plastic bags or containers	Autoclaving or microwaving/hydroclaving followed by shredding or mutilation or combination of sterilization and shredding. Treated waste to be sent to registered or authorized recyclers or for energy recovery or plastics to diesel or fuel oil or for road making, whichever is possible. Plastic waste should not be sent to landfill sites.
White (Translucent)	Waste sharps including metals: Needles, syringes with fixed needles, needles from needle tip cutter or burner, scalpels, blades, or any other contaminated sharp object that may cause puncture and cuts. This includes both used, discarded and contaminated metal sharps.	Puncture-proof, leak-proof, tamper-proof containers	Autoclaving or dry heat sterilization followed by shredding or mutilation or encapsulation in metal container or cement concrete; combination of shredding-cum-autoclaving; and sent for final disposal to iron foundries (having consent to operate from the State Pollution Control Boards or Pollution Control Committees) or sanitary landfill or designated concrete waste sharp pit.
Blue	(a) Glassware: Broken or discarded and contaminated glass including medicine vials and ampoules except those contaminated with cytotoxic wastes. (b) Metallic body implants.	Cardboard boxes with blue-coloured marking Cardboard boxes with blue-coloured marking	Disinfection (by soaking the washed glass waste after cleaning with detergent and sodium hypochlorite treatment) or through autoclaving or microwaving or hydroclaving and then sent for recycling.

Notes:

* Disposal by deep burial is permitted only in rural or remote areas where there is no access to common biomedical waste treatment facility. This will be carried out with prior approval from the prescribed authority and as per the standards specified in Schedule III. The deep burial facility shall be located as per the provisions and guidelines issued by Central Pollution Control Board from time to time.

1. Microbiological waste and all other clinical laboratory waste shall be pre-treated by sterilization before packing and sending to the common biomedical waste treatment facility.
2. Mutilation or shredding must be to an extent to prevent unauthorized reuse.
3. Autoclaving of biomedical waste shall be done at a temperature of not less than 121°C and pressure of 15 pounds per square inch for not less than 60 minutes.

KEY POINTS

- **Sterilization** is a process that **kills** or **removes all** organisms (and their spores) in a material or an object.
- **Disinfection** is a process that **kills** or **removes pathogenic organisms** in a material or an object.
- **Antisepsis** is the application of a chemical agent externally on a **live surface** (skin or mucosa) to destroy organisms or to inhibit their growth (all antiseptics are disinfectants but not vice versa).
- Disinfectants are designed to be used on inanimate objects to kill or destroy disease-producing microorganisms.
- Important agents used in sterilization and disinfection are physical agents (dry and moist heat), and chemical agents (phenols and cresols, halogens, aldehydes, alcohols, vapour-phase disinfectants and surface active disinfectants).

- **Sterilization** is usually achieved by **moist heat** (steam under pressure in an autoclave; most popular), **dry heat** (hot air oven) or gaseous chemicals.
- The sterilization cycle (either in an autoclave or a hot air oven) can be divided into the **heating-up period**, the **holding period** and the **cooling period**.
- The indicators that must be routinely used for checking sterility are **mechanical indicators** (i.e. temperature and pressure gauges of the autoclave), **chemical indicators** and **biological indicators**.
- **Biomedical waste** should be **segregated at source**, since 80% of the waste is non-hazardous and can be disposed of easily into the municipal bin; waste should be segregated in bags of different colours to facilitate appropriate treatment and disposal.



Important Questions

1. Define the terms sterilization, disinfection and antisepsis. Name various agents used for sterilization and discuss the role of hot air oven in sterilization.
2. Discuss the role of moist heat in sterilization.
3. Write short notes on:

(a) Hot air oven	(b) Autoclave
(c) Sterilization by filtration	(d) Sterilization by radiation
4. Name various types of disinfectants and discuss the role of halogens in chemical disinfection.
5. Write short notes on:

(a) Phenols as disinfectants	(b) Halogens as disinfectants
(c) Aldehydes as disinfectants	(d) Alcohols as disinfectants
(e) Vapour-phase disinfectants	(f) Surface active disinfectants
(g) Disinfection of skin	
6. Discuss briefly various tests used for determining efficacy of disinfectants.
7. Name different types of biomedical wastes and discuss in detail the methods of disposal of the same.



Multiple Choice Questions

1. The process of freeing of an article from living organisms including bacterial and fungal spores and viruses is known as:

(a) sterilization.	(b) <i>Brucella</i> .
(b) disinfection.	(c) <i>Salmonella</i> .
(c) antisepsis.	(d) <i>Coxiella burnetii</i> .
(d) all of the above.	
2. Destruction or inhibition of microorganisms in living tissues is known as:

(a) sterilization.	(b) autoclaving.
(b) disinfection.	(c) inspissation.
(c) antisepsis.	(d) pasteurization.
(d) none of the above.	
3. Incineration is an efficient method for:

(a) destroying contaminated materials.	(b) sterilizing points of forceps.
(b) sterilizing points of forceps.	(c) sterilizing scalp blades and needles.
(c) sterilizing scalp blades and needles.	(d) sterilizing all glass syringes.
(d) sterilizing all glass syringes.	
4. Heating in a hot air oven at 160°C for 1 hour is used for sterilization of:

(a) all glass syringes.	(b) <i>Bacillus stearothermophilus</i> .
(b) oils and jellies.	(c) <i>Clostridium perfringens</i> .
(c) swab sticks.	(d) <i>Bacillus cereus</i> .
(d) all of the above.	(e) <i>Clostridium histolyticum</i> .
5. In holder method of pasteurization, milk is kept at:

(a) 63°C for 30 minutes.	(b) 63°C for 60 minutes.
(b) 63°C for 60 minutes.	(c) 72°C for 20 seconds.
(c) 72°C for 20 seconds.	(d) 72°C for 30 seconds.
(d) 72°C for 30 seconds.	
6. Which of the following organisms can survive pasteurization by holder method?

(a) <i>Mycobacterium</i> .	(b) <i>Brucella</i> .
(c) <i>Salmonella</i> .	(d) <i>Coxiella burnetii</i> .
7. Exposure of material to steam at 100°C for 20 minutes on three consecutive days is known as:

(a) tyndallization.	(b) autoclaving.
(b) autoclaving.	(c) inspissation.
(c) inspissation.	(d) pasteurization.
(d) pasteurization.	
8. Which of the following articles can be sterilized in an autoclave?

(a) Dressing material.	(b) Gloves.
(b) Gloves.	(c) Culture media.
(c) Culture media.	(d) All of the above.
(d) All of the above.	
9. Biological control used in an autoclave is the spores of:

(a) <i>Bacillus stearothermophilus</i> .	(b) <i>Clostridium perfringens</i> .
(b) <i>Clostridium perfringens</i> .	(c) <i>Bacillus cereus</i> .
(c) <i>Bacillus cereus</i> .	(d) <i>Clostridium histolyticum</i> .
(d) <i>Clostridium histolyticum</i> .	

10. Which of the following organisms is retained in the fluids filtered by Seitz filter?
 - (a) *Proteus*. (b) *Staphylococcus*.
 - (c) *Clostridium*. (d) None of the above.
11. Gamma rays can be used for sterilization of:
 - (a) plastic syringes.
 - (b) catheters.
 - (c) cannulas.
 - (d) all of the above.
12. Phenol is bactericidal at a concentration of:
 - (a) 0.1%.
 - (b) 0.25%.
 - (c) 0.5%.
 - (d) 1%.
13. Iodophores are mixture of:
 - (a) iodine and surface active agents.
 - (b) iodine and phenols.
 - (c) iodine and aldehydes.
 - (d) iodine and alcohols.
14. Glutaraldehyde is useful for sterilization of:
 - (a) cystoscopes.
 - (b) bronchoscopes.
 - (c) thermometers.
 - (d) all of the above.
15. Infectious diseases transmitted by consumption of raw milk include all of the following, **except**:
 - (a) Q fever.
 - (b) brucellosis.
 - (c) tuberculosis.
 - (d) whooping cough.
16. Heat-sensitive materials (rubber and plastic) and bulky materials (mattresses) can be sterilized by using:
 - (a) dry heat.
 - (b) autoclaving.
 - (c) UV radiation.
 - (d) ethylene oxide.
17. Dry heat damages microbes by:
 - (a) destructive oxidation of essential cell constituents.
 - (b) denaturing proteins.
 - (c) denaturing nucleic acid.
 - (d) none of the above.
18. How is a liquid sterilized without damaging heat-labile proteins in the solution?
 - (a) By boiling.
 - (b) By autoclaving.
 - (c) By passing the liquid through 0.22 μm filter.
 - (d) By passing the liquid through 0.5 μm filter.
19. Which of the following can destroy prions?
 - (a) Exposure to formalin vapour.
 - (b) Treatment with sodium hydroxide and sodium hypochlorite.
 - (c) Irradiation.
 - (d) Boiling.
20. Which of the following bacteria is used as control for ethylene oxide sterilization?
 - (a) *Bacillus subtilis* subsp. *niger*.
 - (b) *Bacillus sterothermophilus*.
 - (c) *Bacillus globigi*.
 - (d) *Bacillus pumilis*.
21. Autoclave is **ineffective** for sterilization of:
 - (a) culture media.
 - (b) dressing material.
 - (c) gloves.
 - (d) powders.
22. Alcohols are **not** active against:
 - (a) vegetative bacteria.
 - (b) fungi.
 - (c) lipid-containing viruses.
 - (d) spores.
23. Alcohols have excellent *in vitro* bactericidal activity against most vegetative Gram-positive and Gram-negative bacteria; however, they are:
 - (a) not fast acting.
 - (b) not sporicidal.
 - (c) not bactericidal.
 - (d) none of the above.
24. What type of filter does a class II biologic safety cabinet use to filter out infectious agents?
 - (a) Millipore filters.
 - (b) HEPA filters.
 - (c) Dust filters.
 - (d) Charcoal filters.
25. Which of the following characteristics should be considered when selecting an antimicrobial agent?
 - (a) Spectrum of activity.
 - (b) Rate of action.
 - (c) Mechanism of action.
 - (d) All of the above.



Answers

- | | | | | | | | | | | | |
|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| 1. a | 2. c | 3. a | 4. d | 5. a | 6. d | 7. a | 8. d | 9. a | 10. d | 11. d | 12. c |
| 13. a | 14. d | 15. d | 16. d | 17. a | 18. c | 19. b | 20. c | 21. d | 22. d | 23. b | 24. b |
| 25. d | | | | | | | | | | | |

Hand Hygiene and Personal Protective Equipment (PPE)

Competency achievement: The student after reading the chapter should be able to:

MI8.6: Describe the basics of infection control.

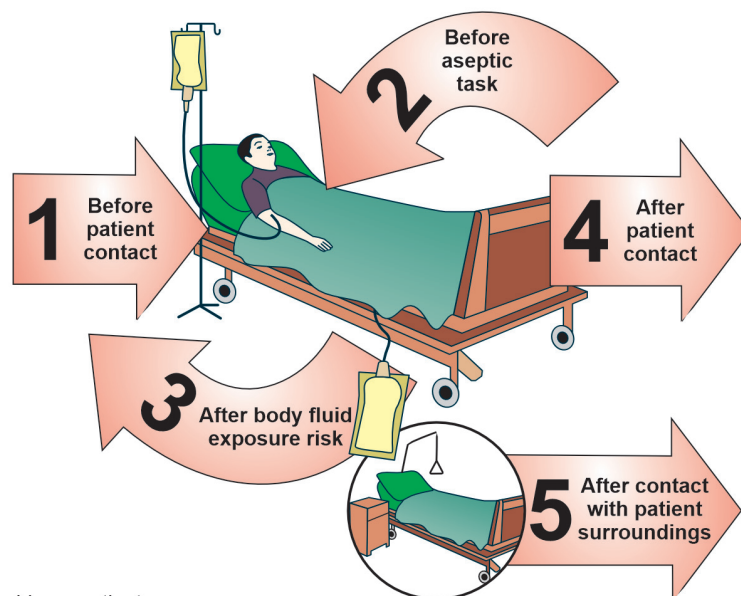
MI8.7: Demonstrate infection control practices and use of personal protective equipment (PPE).

The WHO has published standard guidelines. Situations, when hand hygiene is indicated, are known as “My five moments for hand hygiene”. It includes:

- Before touching a patient,
- Before clean/aseptic procedures
- After body fluid exposure/risk patient
- After touching a patient, and
- After touching the patient’s surroundings.

Clean hands save lives!

Five moments for hand hygiene



1. Before patient contact

Clean your hands before touching a patient.

Examples: Examinations, helping a patient to move, checking name bands.

2. Before an aseptic task

Clean hands before and after an aseptic task.

Examples: Oral care, secretion aspiration, wound care, catheter placement, patient feeding, medication administration.

3. After body fluid exposure risk

Clean your hands immediately after an exposure to a body fluid and after removing gloves.

Examples: After contact with any body fluids to (urine, saliva, sputum, feces, blood, etc.)

4. After patient contact

Clean your hands after any patient contact.

Examples: After activities of daily living, handling of a patient's personal effects, after positioning a patient for an examination or procedure.

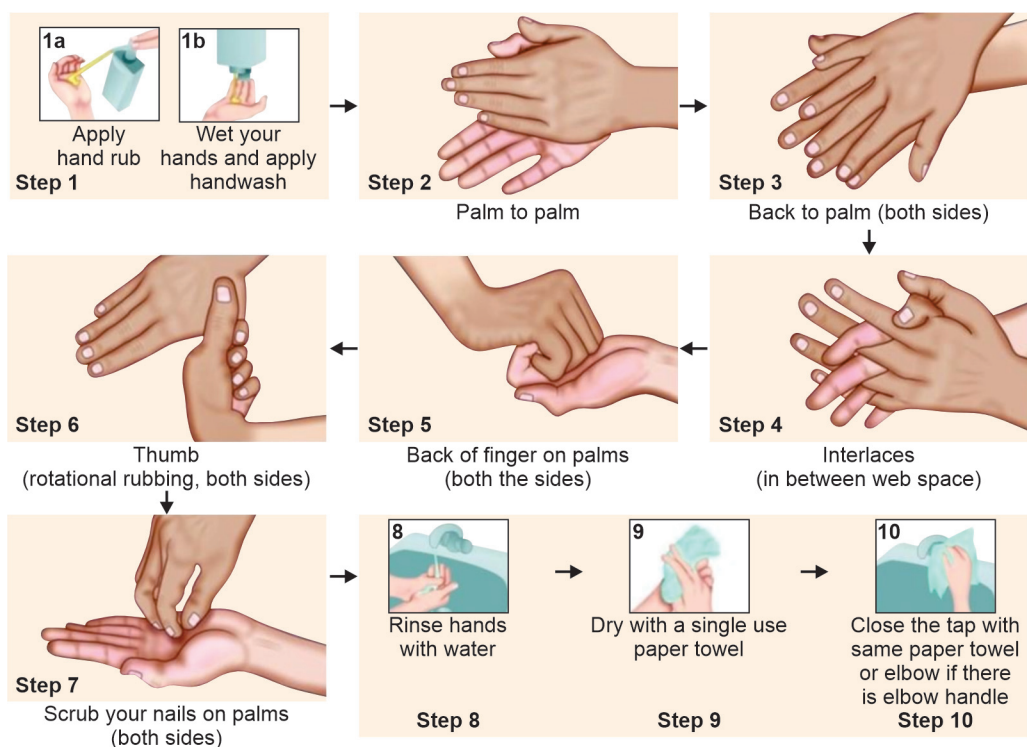
5. After contact with patient surroundings

Clean your hands after you have had contact with a surface that a patient may have touched.

Examples: After cleaning up the patient's bedside and overbed table, making up the bed, moving wheelchairs or walkers.

Steps of Hand Rubbing and Handwashing

Steps of Hand Rubs (Steps 1–7) and Steps of Handwash (Steps 1–10)

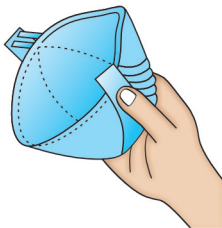


Personal Protective Equipment Kit

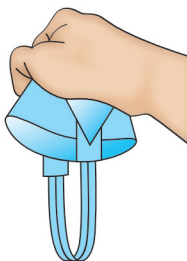


How to wear N95 mask?

N95 Mask



1. Noseclip is located in top panel. Perform the noseclip by gently bending at the center of the panel. Hold respirator in one hand and pull out bottom panel to form a cup



2. Turn respirator over to expose headbands



3. Cup respirator under chin and pull and straps over the head



4. Locate the lower strap below the ear and the upper strap across the crown of the head. Adjust top and bottom panels for a comfortable fit.

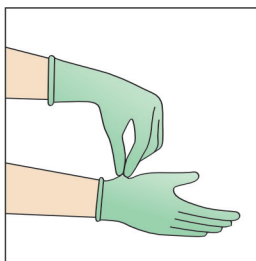


5. Using both hands, mould noseclip to the shape of the lower part of the nose. Pinching the nosepiece using only one hand may result in less effective respirator performance.

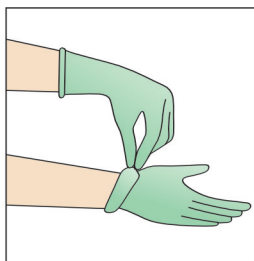


6. The seal of the respirator on the face should be fit—checked prior to wearing in the work area.

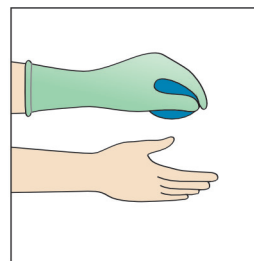
How to remove gloves safely?



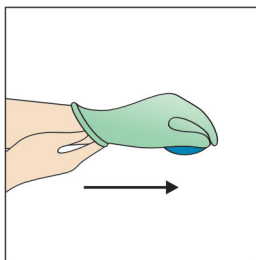
- Grasp the outside of the glove in the wrist area



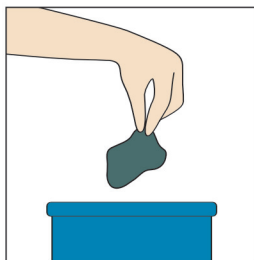
- Peel the glove away from your body, turn it inside-out



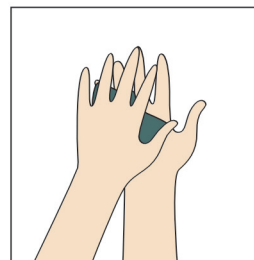
- Hold the inside-out glove in the other hand



- Slide your fingers under the wrist of other glove and peel the glove away from your body



- Dispose the gloves safely



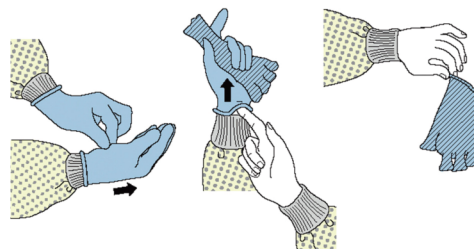
- Wash your hands

Doffing of PPE

There are a variety of ways to safely remove PPE without contaminating your clothing, skin, or mucous membranes with potentially infectious materials. Here is one example. Remove all PPE before exiting the patient room except respirator, if worn. Remove the respirator after leaving the patient room and closing the door. Remove PPE in the following sequence:

1. Gloves

- Outside of gloves are contaminated!
- If your hands get contaminated during glove removal, immediately wash your hands or use an alcohol-based hand sanitizer
- Using a gloved hand, grasp the palm area of the other gloved hand and peel off first glove
- Hold removed glove in gloved hand
- Slide fingers of ungloved hand under remaining glove at wrist and peel off second glove over first glove
- Discard gloves in a waste container



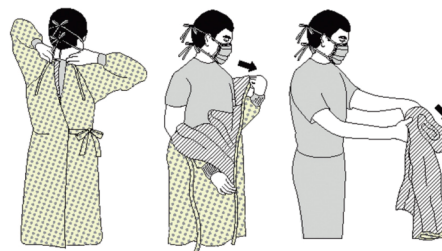
2. Goggles or face shield

- Outside of goggles or face shield are contaminated!
- If your hand get contaminated during goggle or face shield removal, immediately wash your hands or use an alcohol-based hand sanitizer
- Remove goggles or face shield from the back by lifting head band or ear pieces
- If the item is reusable, place in designated receptacle for reprocessing. Otherwise, discard in a waste container



3. Gown

- Gown front and sleeves are contaminated!
- If your hands get contaminated during gown removal, immediately wash your hands or use an alcohol-based hand sanitizer
- Unfasten gown ties, taking care that sleeves do not contact your body when reaching for ties
- Pull gown away from neck and shoulders, touching inside of gown only
- Turn gown inside out
- Fold or roll into a bundle and discard in a waste container

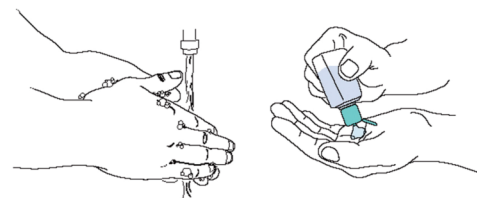


4. Mask or respirator

- Front of mask/respirator is contaminated— Do Not Touch!
- If your hands get contaminated during mask/respirator removal, immediately wash your hands or use an alcohol-based hand sanitizer
- Grasp bottom ties or elastics of the mask/respirator, then the ones at the top, and remove without touching the front
- Discard in a waste container



5. Wash hands or use an alcohol-based hand sanitizer immediately after or removing all PPE



Perform hand hygiene between steps if hands become contaminated and immediately after removing all PPE



DISCUSSION

1. Why is hand hygiene important?

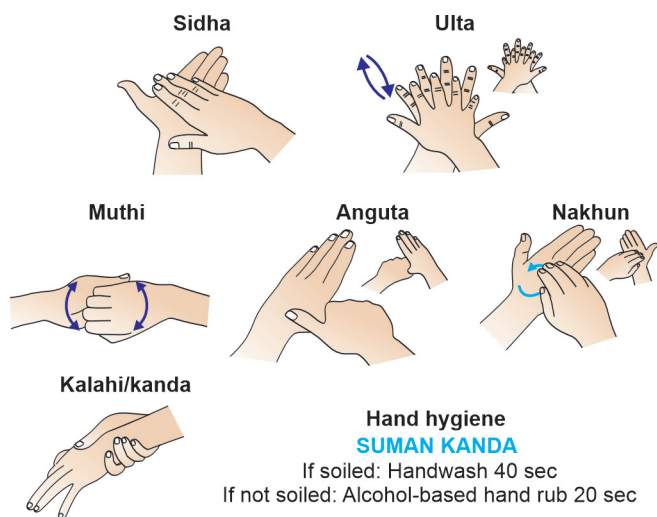
Hand hygiene is important to prevent

- Cross contamination.
- The spread of disease causing germ through hands.
- Spread of healthcare associated infections.

2. What is mnemonic for handwashing steps?

SUMAN-K

S-sidha U-ulta M-muthi
A-anguta N-nakhun K-kalahi/kanda



3. Mention the steps of donning of PPE and doffing of PPE.

Donning sequence	Doffing sequence
Handwash	Hand rub
↓	↓
Cap	Outer gloves
↓	↓
Shoe cover	Hood
↓	↓
Hand rub	Coverall/gown
↓	↓
Inner glove	Shoe cover
↓	↓
Coverall/gown	Hand rub (gloved hand)
↓	↓
Mask (surgical or N95)	Goggles
↓	↓
Goggles	Mask (surgical or N95)
↓	↓
Hood	Cap
↓	↓
Outer gloves	Inner glove
	↓
	Handwash

4. Explain the fit check for N95 respirators.

HCWs must perform fit checking every time they put on a N95 respirator to ensure if it is properly fitted and functional.

- **Placement:** The respirator is placed on the face and tied over the head and at the base of the neck.
- **Sealing:** N95 mask is compressed to ensure a seal across the face, cheeks and bridge of the nose.
- **The positive pressure seal** of the N95 mask is checked by gently exhaling. If air escapes, the N95 mask needs to be adjusted.
- **The negative pressure seal** of the N95 mask is checked by gently inhaling. If the N95 mask is not drawn in towards the face, or air leaks around the face seal; the N95 mask is readjusted and the process is repeated. If still not proper, then its respirator should be checked for any defect or damage.

5. What precautions should be taken while using the N95 mask?

- Discard N95 masks when contaminated with blood, respiratory or nasal secretions, etc.
- Consider use of a cleanable face shield (preferred) over an N95 respirator and/or other steps (e.g. masking patients) to reduce surface contamination.
- Perform hand hygiene before and after touching or adjusting the N95 mask.
- **Extended use of N95 mask:** Refers to wearing the same N95 respirator for repeated close contact encounters with several patients, without removing the respirator between patient encounters; as long as they are functional well (up to 8 hours).

Bacterial Genetics

Competency achievement: The student after reading the chapter should be able to:

MI1.6(a): Describe the mechanism of drug resistance.

Genetics is the study of genes, their structure and function, heredity and variation. Like other organisms, bacteria too obey the laws of genetics and breed true. However, a small proportion of their progeny exhibits variations in some properties. The genetic information in bacteria, as in other cells, is contained in the specific sequence of nucleotides in the cell's deoxyribonucleic acid (DNA). The DNA acts as a template for its own replication so that at the time of cell division two copies are available. It also acts as a template (Fig. 7.1) for transcription of messenger RNA (mRNA) which is then translated, by ribosomes into particular polypeptide (DNA → RNA → polypeptide). This is the **central dogma of molecular biology**. DNA is the storehouse of all information for protein synthesis. However, in 1970, Baltimore and Temin showed that the retroviruses (RNA viruses) encode an RNA-dependent deoxyribonucleic acid polymerase (reverse transcriptase) and replicate through a DNA intermediate. The DNA copy of the viral genome is then integrated into the host chromosome to become a cellular gene.

STRUCTURE OF DNA

DNA molecule is composed of two chains of nucleotides wound together in the form of double helix (Fig. 7.2). Each chain has a backbone of alternatively arranged molecules of deoxyribose sugar and phosphates. To each deoxyribose sugar is attached one of the four nitrogenous bases, i.e. pyrimidines (thymine and cytosine) and purines (guanine and adenine). Hydrogen (H) bond unites two nitrogenous bases of opposite strands, thus making it double-stranded. Adenine always binds to thymine and guanine to cytosine. Therefore, adenine is complementary to thymine and guanine to cytosine.

STRUCTURE OF RNA

Structure of RNA is similar to that of DNA with two differences:

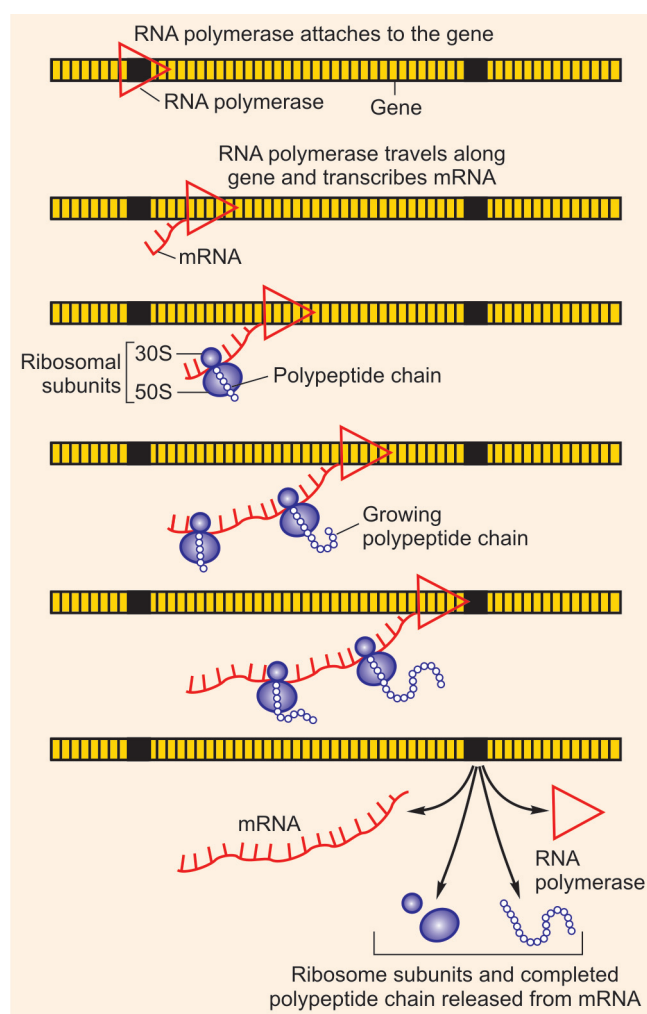


Fig. 7.1: Synthesis of polypeptide.

1. It has sugar ribose instead of deoxyribose.
2. It has nitrogenous base uracil in place of thymine in DNA.

On the basis of structure and function RNA is of three types:

1. **Ribosomal RNA (rRNA):** This is RNA of ribosomes. It plays a basic role in the synthesis of proteins by providing a place for the specific selection, arrangement and joining of amino acids.

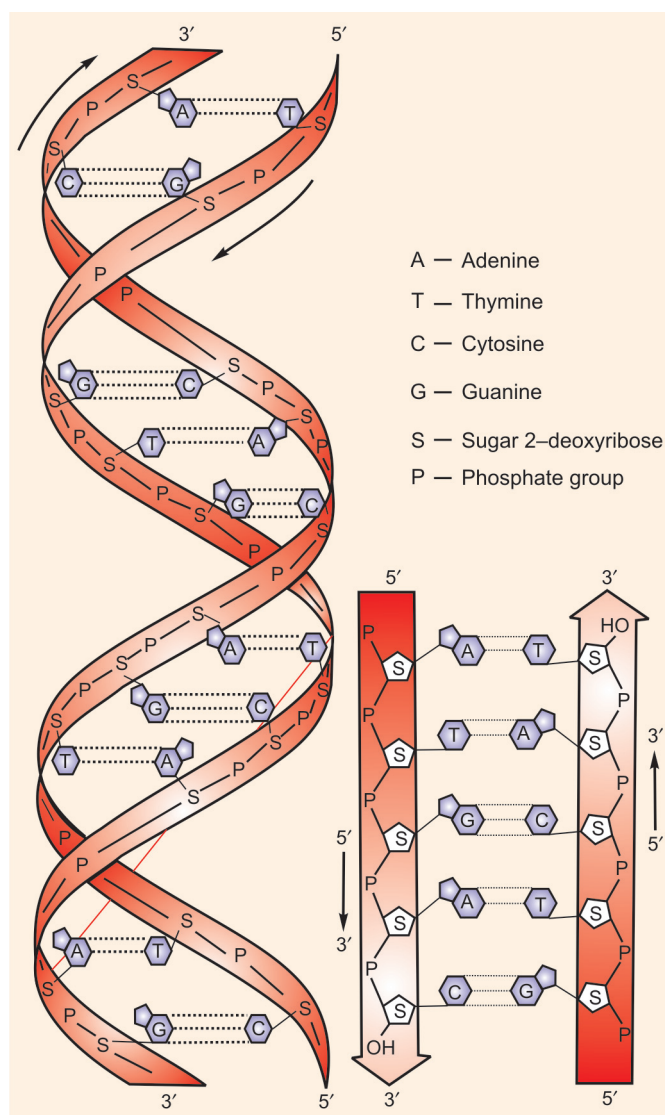


Fig. 7.2: Watson and Crick model of DNA.

2. **Transfer RNA (tRNA):** This RNA has a specific terminal nucleotide sequence that enables it to accept a single molecule of an activated amino acid and transfer it to a ribosome.
3. **Messenger RNA (mRNA):** This RNA is formed by an enzyme RNA polymerase under the direct influence of DNA. Since DNA acts as a template for synthesis of mRNA, therefore, the bases in the two will be complementary to each other. Adenine, guanine, cytosine and uracil in mRNA will be complementary to thymine, cytosine, guanine and adenine, respectively in DNA.

Codon

Genetic information is stored in DNA as a code. The unit of code is known as codon. It consists of a sequence of three bases. Therefore, code is triplet. Each codon specifies or codes for a single amino acid, but more than one codon may exist for a single amino acid. Thus codon AGA codes for arginine but the codons AGG, CGU, CGC, CGA and CGG also code for the same amino acid. Therefore, code is

degenerate. Three codons UAA, UAG and UGA do not code for any amino acid and are known as **nonsense codons**. They act as punctuation marks, terminating the message for synthesis of a polypeptide.

A segment of DNA carrying a number of codons specifying for a particular polypeptide is known as **cistron** or **gene**. A large number of genes constitute a **locus** and a large number of loci constitute cell **genome**. Therefore, DNA can be compared with a book of information. Letters represent nucleotides, words represent codons, sentences represent genes, paragraphs represent loci and entire book as DNA molecule or **cell genome**.

Most bacteria contain enough DNA to code for 1,000–3,000 different types of polypeptide chains, i.e. 1,000–3,000 genes. DNA molecule of *Escherichia coli* is 1,000–1,300 μm long. Since, it is much longer than the length of the bacteria, therefore, it is coiled and tightly packed up in a bundle resembling a skein of woollen thread. The length of DNA is usually expressed as kilobases (1 kb = 1,000 base pairs).

EXTRACHROMOSOMAL GENETIC ELEMENTS

Plasmid

In addition to chromosomal DNA, bacteria may also possess extrachromosomal genetic material known as plasmid. It may be defined as a small extrachromosomal piece of genetic material that can replicate autonomously (independent replicans) and can maintain in the cytoplasm of a bacterium for many generations. It consists of a circular piece of double-stranded DNA. It is not essential for the normal life and function of the host bacterium though it may confer on it additional properties such as drug resistance, bacteriocin production, toxigenicity, etc. which may confer on the host bacterium survival advantage under appropriate conditions. In addition, it may contain genetic information for controlling its own replication and ensuring segregation of one copy into each daughter cell at cell division. Plasmid contains 50–100 genes. By their ability to transfer genes from one cell to another, plasmids have become important vectors in genetic engineering.

Episome

Plasmid DNA (extrachromosomal) may get integrated into host cell genome. In this state it is often known as episome. But both these terms are frequently used synonymously. Some plasmids confer on the host cell maleness or ability to conjugate (bacterial equivalent of sexual mating in higher organisms). Following conjugation, the recipient acquires the plasmid and in turn becomes male or donor cell.

GENOTYPIC AND PHENOTYPIC VARIATIONS

The characteristics expressed by a cell in a given environment are referred to as its **phenotype** (*pheno*—display) and the collection of genes encoding these characteristics, the **genotype**. Bacteria, in general, are very adaptable and may

alter their phenotype in response to environmental change while the genotype remains unchanged. Not all the genes of the bacterial cell are expressed all the time. For example, typhoid bacillus is normally flagellated but when grown on phenol agar, the flagella are not synthesized, but when subcultured from phenol agar into nutrient broth flagellated cells appear.

Another example of environmental influence is the synthesis of an enzyme β -galactosidase, necessary for lactose fermentation to its constituent sugars—glucose and galactose, in *Escherichia coli*. This organism possesses genetic information for synthesis of the enzyme, but it is produced by the bacteria only when lactose is present in the growth medium. This enzyme is not synthesized if *E. coli* is grown in medium containing glucose only. Such enzymes, which are synthesized only when induced by substrate, are called **induced enzymes**, while those enzymes, which are synthesized both in the presence or absence of the substrate, are known as **constitutive enzymes**. Regulation of enzyme induction illustrates the economy of nature, the enzyme being produced only when appropriate substrates are present.

- **Phenotypic variations** are influenced by environment, are temporary and are not heritable.
- **Genotypic variations** are stable, heritable and are not influenced by the environment. These are due to mutation or by any of the methods of genetic transfer such as transformation, transduction, lysogenic conversion and conjugation.

LAC OPERON (GENE REGULATION)

Operon concept was proposed by Jacob and Monod. Lactose fermentation requires three enzymes— β -galactosidase, galactoside permease and transacetylase coded by structural genes *Lac-z*, *Lac-y* and *Lac-a* of lac operon, respectively (Fig. 7.3). Regulatory gene in this case is *Lac-i* which codes for a repressor. It is a protein molecule which can combine with either operator region on the chromosome or with the inducer (lactose). Between *Lac-i* and structural *Lac* genes lie promoter and operator regions. For transcription of mRNA for enzyme synthesis, the RNA polymerase has to attach to promoter region and travel along structural genes sequence. Operator region lies between the promoter and structural genes.

In resting stage when lactose (inducer) is not present in the medium, repressor molecule is bound to the operator, preventing the passage of RNA polymerase from promoter to the structural genes. The repressor molecule has an affinity for lactose, in the presence of which it leaves the operator region free enabling the transcription to take place. Lactose thus acts both as an inducer and the substrate for β -galactosidase. When lactose present is completely metabolized, the repressor again attaches to the operator, switching off transcription.

MUTATION

It is a random, undirected, heritable variation caused by an alteration in the nucleotide sequence at some point of the

DNA of the cell, which may be due to addition, deletion or substitution of one or more bases. Mutations occur spontaneously at fairly constant rates, usually in the range of one per 10^2 – 10^{10} cell divisions. A large colony of bacteria contains about 10^9 cells, all derived from a single organism by repeated cell divisions. It may be realized that after 10^9 cell divisions, many thousands of different mutations will have occurred, affecting many of the genes in the cell. Similarly, in every infected patient, a variety of mutants arise spontaneously.

Mutants will outnumber and overgrow, if their new character makes them better fitted to grow under the prevailing conditions in the culture medium or host tissue than the parental bacteria. This is known as **selection of mutants**. An antibiotic resistant mutant will outgrow the sensitive parental bacteria in a culture medium containing antibiotic or in the body of the patient receiving antibiotic therapy. **A mutant with altered surface antigens will escape deleterious effect of immunity developed previously.**

Though mutation occurs spontaneously, its frequency can be artificially increased by several mutagenic agents such as nitrogen mustard, acriflavine, mitomycin-C, 5-bromouracil and 2-aminopurine.

Types of mutation

Missense mutation

When triplet code is altered so that it codes for an amino acid different from that normally located at a particular position in the protein, this is known as missense mutation.

Nonsense mutation

Deletion of a nucleotide within a gene may cause premature polypeptide chain termination by producing a nonsense codon. This is known as nonsense mutation.

Transversion

It is substitution of a purine for a pyrimidine and vice versa in base pairing.

Conditional lethal mutation

When mutations involve vital functions so that the mutants are non-viable, these are known as lethal mutations. An important type of lethal mutation is conditional mutation. A conditional lethal mutant may be able to live under certain permissive conditions but not under other or non-permissive conditions. The commonest type of conditional lethal mutant is **temperature-sensitive (ts) mutant** which is able to live at 35°C (permissive temperature) but not at 39°C (restrictive temperature).

Though, mutations are taking place all the time, most mutants go unrecognized as the mutation may involve minor function or it may be lethal. Mutation can be best recognized when it involves a function which can be readily observed by experimental methods like alteration in colonial morphology, pigmentation, alteration in cell surface antigens, sensitivity to bacteriophages or bacteriocins, loss of ability to produce capsule or flagella, loss of virulence and change in biochemical characters.

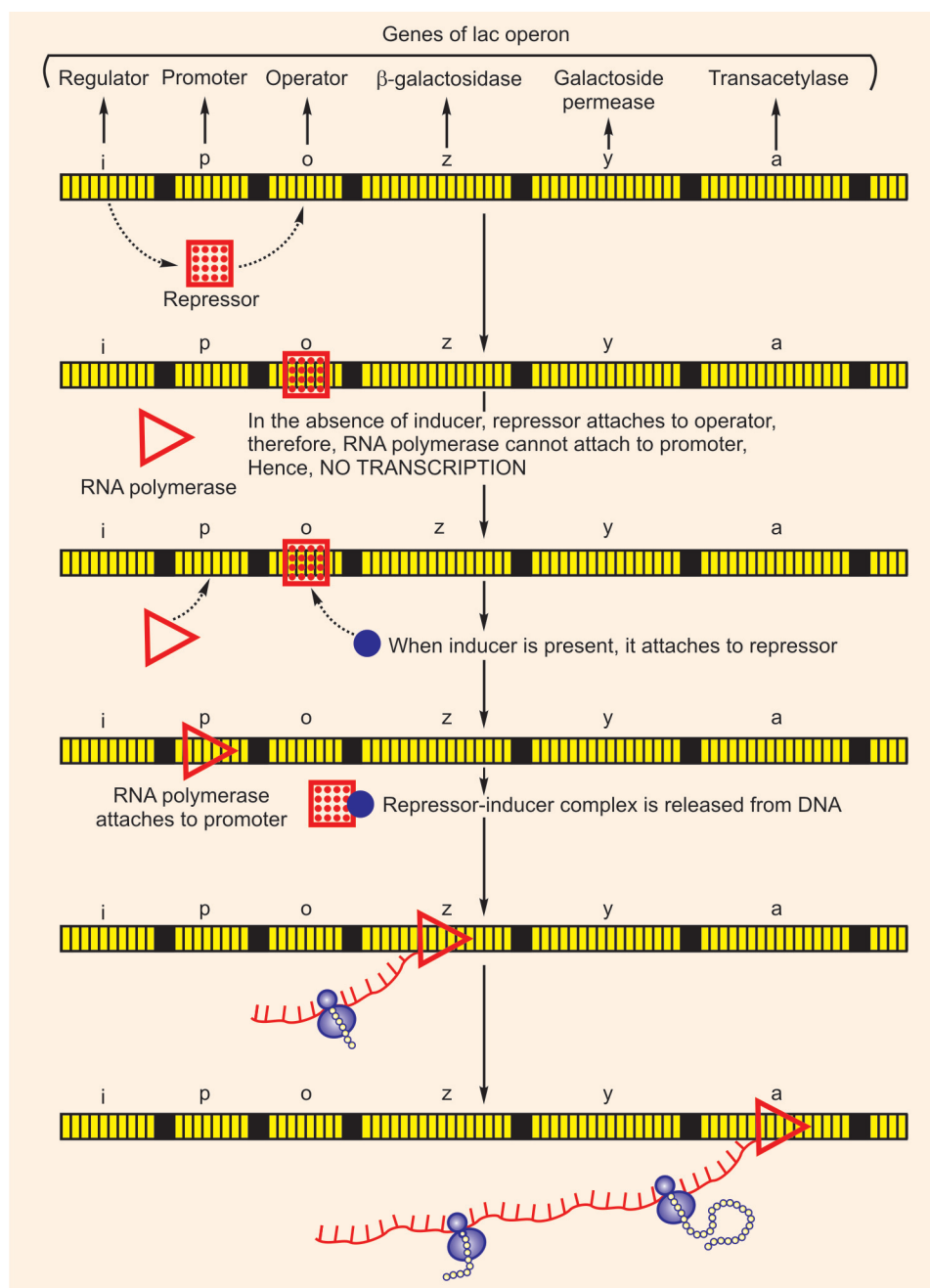


Fig. 7.3: The lac operon of *Escherichia coli*.

The practical importance of bacterial mutation is mainly in the field of drug resistance and development of live vaccines. Some organisms have been subcultured in the laboratory for many generations until they lost their virulence for man (e.g. BCG vaccine). This is known as **live attenuated vaccine**.

Demonstration of mutation in laboratory

Mutations can be demonstrated in the laboratory by fluctuation test and replica plating:

Fluctuation test

The proof that bacteria undergo spontaneous mutation independent of environment was provided by Luria and

Delbruck (1943) by fluctuation test. They studied bacteriophage-sensitive *E. coli*. Samples from a single large volume culture, and small volume cultures were plated on solid media containing bacteriophage. The mutant bacteria (bacteriophage-resistant) formed colonies. Wide fluctuations in the number of bacteriophage-resistant colonies in small volume cultures as compared to a single large volume culture were observed (Fig. 7.4). Statistically, this indicated that mutations occurred randomly in separate small volume cultures, some early some late, resulting in wide fluctuation. In the large volume cultures, fluctuations were within the limits of sampling error. However, because of the complicated statistical interpretation, this experiment was not widely appreciated.

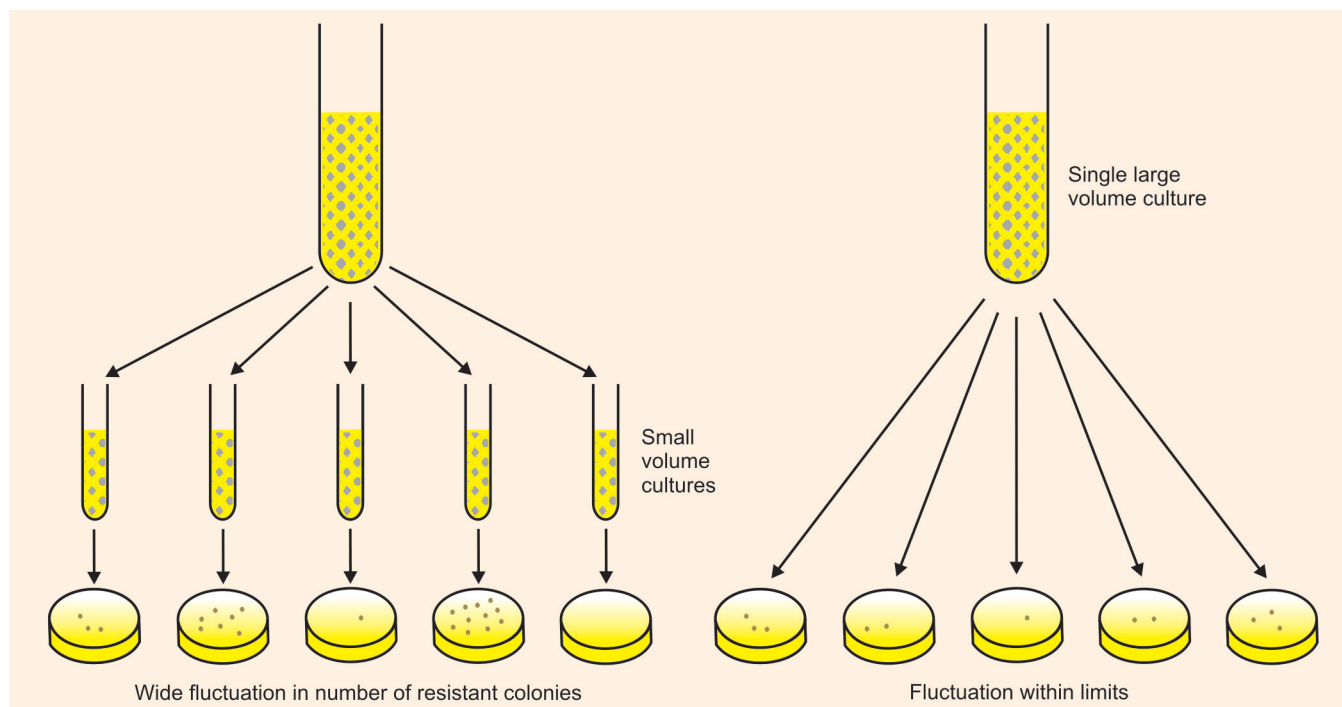


Fig. 7.4: Fluctuation test.

Replica plating method

Using a velvet template, Lederberg and Lederberg (1952), transferred inocula from colonies on a master plate, onto a number of other plates, retaining the relative positions of the colonies in all the plates. By replica plating on culture plates with and without bacteriophage, they were able to demonstrate that bacteriophage-resistant mutants appeared without ever having had contact with the selective agent (Fig. 7.5).

ACQUISITION OF NEW GENES

Change in the genome of a bacterium may be due to mutation in the organism's own DNA or to acquisition of DNA from an external source. Transmission of genetic material may take place by:

1. Transformation
2. Transduction
3. Lysogenic conversion
4. Conjugation

1. Transformation

Acquisition of DNA by a bacterium from its environment and incorporation in its genome is known as transformation. It is perhaps the most important mechanism of genetic exchange for certain bacterial species, notably *Streptococcus pneumoniae*, *Bacillus subtilis*, *Haemophilus influenzae* and *Neisseria gonorrhoeae*. For transformation to occur DNA must have been derived from a closely related strain, since a piece of DNA can undergo recombination with chromosome only when there is adequate nucleic acid homology. Transformation was first demonstrated by Griffith in 1928. *S. pneumoniae* in capsulated form is an extremely virulent

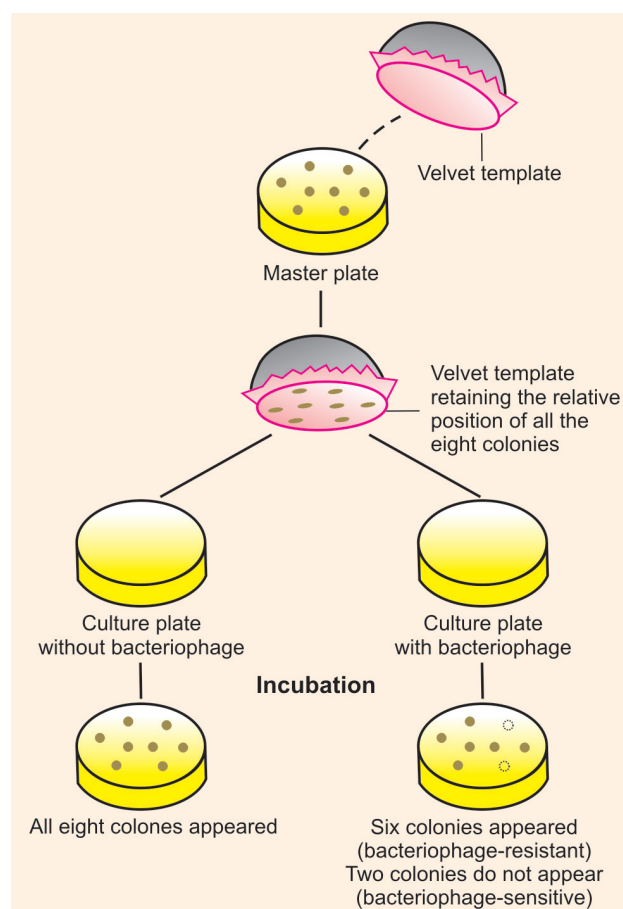


Fig. 7.5: Replica plate method.

organism for mice, whereas non-capsulated variants are avirulent. The virulence of the organism is due to polysaccharide capsule.

Mutant pneumococci that have lost their ability to synthesize this capsule arise spontaneously. These show rough (R) colonies on blood agar as compared to smooth (S) colonies of capsulated form of *Pneumococcus*. Griffith mixed live non-capsulated cells that had originally produced a capsule of one antigenic type with heat-killed smooth cells of a different capsular type. Neither preparation alone caused disease in mice, but mixture of the two preparations was lethal and Griffith was able to isolate from blood of the mice live pneumococci having a capsule of the same antigenic type as that of heat-killed cells. It was later shown that **DNA was the transforming principle** that was released from the heat-killed bacteria and was able to confer upon a live recipient cell the ability to produce a new type of capsule. The DNA that was taken up by the rough cells supplied the genetic information needed to make the missing enzyme needed for capsule synthesis.

2. Transduction

Transfer of a portion of DNA from one bacterium to another by bacteriophages (phages) is known as transduction. It may be generalized when it involves any segment of donor DNA or it may be restricted when a specific bacteriophage transduces only a particular portion of DNA.

Generalized transduction

Phages are viruses that multiply in bacteria. They carry their genetic information inside a protein coat. During assembly each phage head is normally filled with a phage genome, but sometimes an occasional phage particle is formed at a frequency of 1 in 10^6 whose head has been accidentally filled with a similar length of host cell DNA. This is known as 'packing error'. When such a particle attaches to a second cell the DNA that enters the cell is not phage DNA capable of replicating and lysing the cell but a short segment of chromosome from first host, thus bacterial genes have been transduced by the phage into a second cell.

Since these phages pick up any portion of the host chromosome, they can transduce any gene. Each transducing phage can pick up a piece of bacterial DNA about the same size as its normal phage genome. Genes can be transduced only between fairly closely related strains because bacteriophage attacks a limited range of organisms with the same surface receptors. Transduction is not confined to transfer of chromosomal DNA. Plasmids or episomes may also be transduced. **The plasmid determining penicillin resistance in staphylococci is transferred from cell-to-cell by transduction.**

Restricted transduction

Bacteriophages that lyse the host cell are known as **virulent phages**, however, **temperate phage** may get incorporated into the host genome and divide with it. These cells are known as **lysogenic**. Molecular basis of lysogeny has been extensively studied in λ phage of *E. coli*. When infected with this phage majority of the cells go into lytic cycle while in a small proportion of them, they get incorporated into host cell genome (**lysogeny**). In some lysogenic cells lytic cycle

is resumed. The **prophage** (integrated phage DNA) is excised and codes for viral proteins and DNA. In a small proportion of cells prophage is excised inaccurately so that a neighbouring portion of bacterial DNA is also removed.

Since phage head can contain only a standard amount of DNA, a transducing phage contains a few bacterial genes at one end of its DNA and lacks a few phage genes at the other end, i.e. **phage genome is defective**. When such a piece of DNA is transduced into a second cell the defective phage can still integrate into its normal site on the chromosome. The added bacterial genes are reproduced in the progeny of the recipient bacterium. Since the temperate phage has a specific insertion site it can pickup and transduce only a short length of DNA containing a few genes on either side of this site. This is known as restricted transduction. λ phage is always inserted between the genes for galactose utilization (*gal*) and biotin synthesis (*bio*) and thus can transduce either *gal* or *bio* genes.

3. Lysogenic conversion

In the lysogenic bacteria the prophage behaves as an additional segment of the bacterial chromosome, coding for new characters. This process by which prophage DNA confers additional genetic information to the host cell is known as **lysogenic or phage conversion**. An important example of lysogenic conversion is of *Corynebacterium diphtheriae* by β -phage. Only those organisms which are lysogenic produce diphtheria toxin because **tox gene** coding for the production of **diphtheria toxin** is present on the phage DNA. The cells that lose the phage, lose toxin production. Another example of lysogenic conversion is the production of **dick toxin** by *Streptococcus pyogenes*.

Abortive transduction

Sometimes transduced DNA fragment does not integrate into the recipient chromosome, perhaps because of the presence of only a limited degree of homology. However, the transduced fragment can express its genetic information but cannot replicate. They remain only in one progeny cells following division. This process is known as abortive transduction.

4. Conjugation

It is the transfer of DNA that occurs during contact between bacterial cells. This mechanism is much more efficient than transformation or transduction. Transfer of DNA by conjugation is very common among Gram-negative bacteria, but it is rare in Gram-positive bacteria. **It is a major mechanism of transfer of drug resistance and can occur among unrelated genera.**

Conjugation was first described by Lederberg and Tatum in 1946 in *E. coli* K12 strain and has been most extensively studied in this strain. The donor status of a bacterial cell is determined by the presence of a plasmid which codes for **sex pilus**. It is 1–2 μm in length. The tip of the pilus attaches to the surface of a recipient cell and holds the two cells together. The two strands of plasmid separate. One strand enters the recipient bacterium, probably along the sex pilus,

while one strand remains in the donor. Each strand then makes a complementary copy (Fig. 7.6). It is probable, but not absolutely certain, that transfer actually occurs through the pilus. Alternately, the pilus could act as a mechanism by which the donor and recipient cells are drawn together. As donor ability is dependent upon having a copy of the plasmid, therefore, the recipient strain becomes converted into a donor. It can, in turn, conjugate with other recipient cells.

Stability of inheritance of plasmids varies from one plasmid to another. Some are lost very easily while others are very stable indeed and rarely, if ever, lost. Some plasmids can be eliminated from the host cell artificially (cured) by treatment with one of a variety of chemical agents (**curing agents**). These include acridine orange, sodium dodecyl sulphate and ethidium bromide.

Fertility (F) factor or F plasmid

F factor or F plasmid is a transfer factor that contains the genetic information necessary for the synthesis of the sex pilus and for self transfer but it does not possess other identifiable genetic markers such as drug resistance. Cells that possess one or more copies of the F plasmid are called F^+ and the cells lacking the F plasmid are called F^- . F^+ cells have no distinguishing features other than their ability to mate with F^- cells and render them F^+ (Fig. 7.6).

Certain *E. coli* strains contain an F plasmid that has become permanently integrated into the cell's chromosome. Such cells are able to transfer chromosomal genes to recipient

cell with high frequency and are known as **Hfr (high frequency recombinant) cells**. F plasmid may revert from Hfr state to free state. Sometimes it may carry with it some chromosomal genes from near the site of its attachment leaving a part of its own DNA from other end in the donor chromosome (Fig. 7.7). Such F factor possessing chromosomal genes is known as **F' (F prime) factor**. When an F' cell mates with a recipient cell, it transfers, along with the F factor, the host genes incorporated with it. This process of transfer of host genes through F factor resembles transduction and is known as **sexduction**.

Resistance plasmid or R plasmid or R factor

It consists of two parts—resistance transfer factor and resistance determinants (RTF + *r*). RTF is responsible for conjugal transfer and *r* determinants code for resistance against various drugs. As many as eight or more *r* determinants may be carried on each plasmid. Therefore, resistance to eight or more drugs may be transferred simultaneously. Sometimes RTF dissociates from *r* determinants. Those plasmids which lack RTF, i.e. possess only *r* determinants are known as **non-conjugative** or **non-self-transmissible plasmids** but they still code for drug resistance. Those plasmids which possess both RTF and *r* determinants are known as **conjugative** or **self-transmissible plasmids**. Non-conjugative plasmids can, however, be transferred by adding another strain possessing transfer factor like colicin plasmid or by bacteriophages.

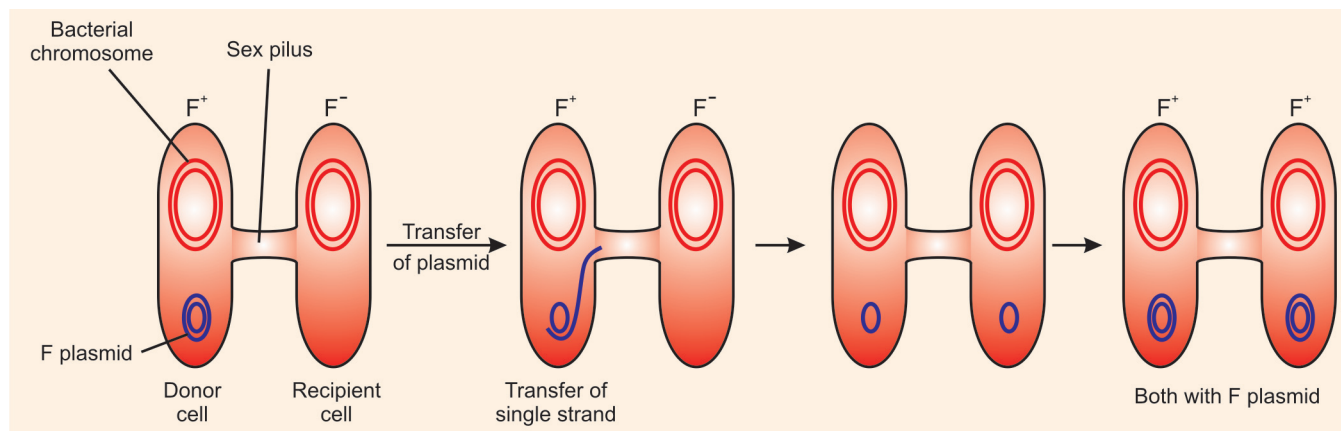


Fig. 7.6: Process of conjugation.

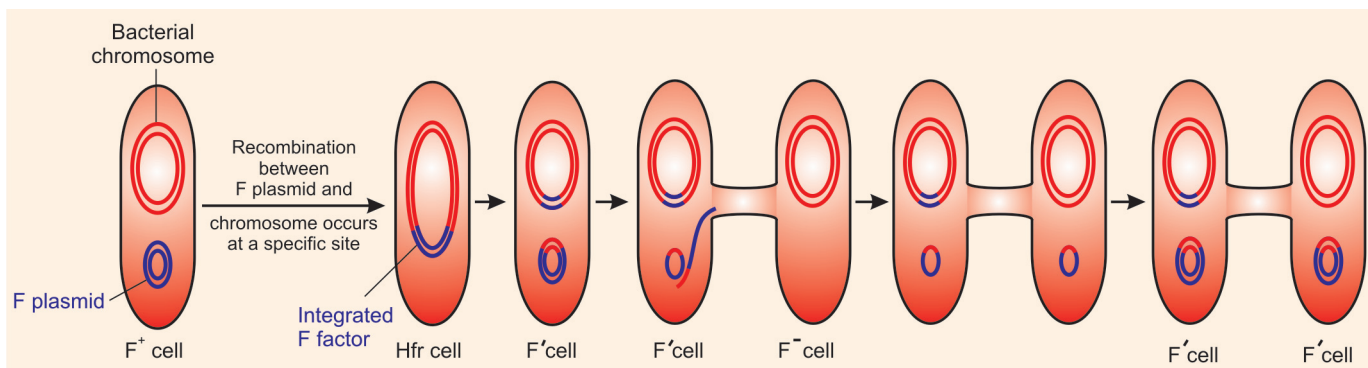


Fig. 7.7: Process of sexduction.

Colicinogenic (col) factor

Several strains of coliform bacteria produce colicins. These are antibiotic-like substances that are specifically and selectively lethal to other enterobacteria. Since similar substances are also produced by other than coliforms, e.g. pyocin by *Pseudomonas aeruginosa* and diphthericin by *Corynebacterium diphtheriae*, therefore, the name bacteriocin has been given to this group of substances. The specificity of action of bacteriocins enables intraspecies classification of certain bacteria, e.g. colicin typing (*Shigella sonnei*), klebocin typing (*Klebsiella* spp.), proticin typing (*Proteus* spp.), and pyocin typing (*Pseudomonas* spp.).

Colicin and klebocin production is known to be mediated by col, and klebocin plasmids (Arora and Chugh, 1982), respectively.

ANTIBIOTIC RESISTANCE

Antibiotic resistance in bacteria may either be intrinsic or acquired. **Intrinsic resistance** means that the bacteria were resistant to the antibiotic even before the antibiotic was introduced. **Acquired resistance** means that a bacterium that was previously sensitive to an antibiotic has now turned resistant. It is the acquired resistance that is of great importance because it would result in treatment failure as well as potential dissemination of resistance to other bacteria.

The physiological mechanisms of antibiotic resistance include:

- Inactivation of the antibiotic by enzymes produced by the bacteria.
- Alteration of target proteins such that the antibiotic does not bind or binds with decreased affinity.
- Alteration of the membrane which decreases the permeability of the antibiotic.
- Active efflux of the antibiotic.
- Development of alternate metabolic pathway to bypass the action of antibiotic.

With antibiotics, as previously with sulphonamides, resistance began to arise. At first, organisms were encountered with low-level resistance to penicillin, and infections caused by these could be treated with larger doses of the antibiotics, because the low-level resistance was due to mutation which decreases the cell permeability to the antimicrobial agent and if larger doses are given then effective concentration of the drug can enter the bacterial cell. In time, however, highly resistant strains emerged which could not be therapeutically controlled.

Genetic basis of resistance

The initial low-level resistant microbial strains carried genes for resistance on the **chromosome**. These were soon replaced by higher-level resistant types, principally harbouring **plasmids**. Plasmid-mediated drug resistance is seen in various pathogenic and commensal bacteria of man and animals, e.g. *E. coli*, *Klebsiella pneumoniae*, *Proteus*, *Salmonella*, *Shigella* and *Pseudomonas*. Transfer of drug resistance occurs readily *in vitro*. It also occurs *in vivo* but in

normal intestines it is inhibited by several factors like anaerobic conditions, bile salts, alkaline pH and abundance of anaerobic Gram-positive bacteria minimising the chances of contact between donor cells and suitable recipient cells. But in intestines of persons on oral antibiotic therapy, transfer occurs readily due to the destruction of the sensitive normal flora and the selection pressure provided by the drug.

Transferable drug resistance involves all antibiotics in common use. Bacteria containing R plasmid can spread from animal-to-man. Therefore, indiscriminate use of antibiotics in man and animals or in animal feed can increase the spread of plasmid-mediated drug resistance in the community. Because of the misuse and overuse of antibiotics in the hospitals it is said '*hospital is the heaven for drug resistant bacteria*'.

Some chromosomal-mediated resistances remain a therapeutic problem, namely, those responsible for resistance to methicillin, rifampicin, nalidixic acid and isoniazid. However, most resistances of concern are associated with R plasmids. While plasmids may be the vectors of the resistance genes, the genes may themselves be located on discrete movable DNA elements called **transposons**. These are very often on plasmids, but have the ability to 'hop' from plasmid to plasmid or from plasmid to chromosome. In many bacteria, such as staphylococci, bacteriophages may also be important vectors for transposon spread.

Transposons may carry single or multiple resistances. The transposon can enter and remain stable in different species even if its entry vector (e.g. plasmid or phage) is lost, since the resistance transposon can be incorporated in a stable resident plasmid or the chromosome of the new host. The discovery of transposable resistance provided the basis for understanding the rapid spread of resistance markers throughout the bacterial kingdom and helped to explain why different genera seemed to have 'evolved' similar genes of resistance.

Bacteria resist antimicrobial agents by various mechanisms. **Chromosomal resistance**, except when resulting from transposon insertions, is generally mediated by a single low-level, non-enzymatic mechanism. In many organisms, these resistances seem related to decreased permeability, e.g. resistance in meningococci, mycobacteria, pneumococci and methicillin resistance in *S. aureus*. Exceptions are the chromosomally located degradative enzymes for the β -lactam antibiotics and the chromosomal-mediated resistance to rifampicin (via resistant RNA polymerases) and nalidixic acid (via resistant DNA gyrases).

In general, **the plasmid- or transposon-mediated resistances** are of higher level and involve more active processes. Some plasmid-mediated resistance to penicillin and to chloramphenicol seems to involve decreased permeability, but the exact mechanism is not yet defined. Many plasmid-mediated resistances involve extracellular inactivation of the drug by enzymatic modification or degradation of its active element. In this category are the enzymes (β -lactamases) which destroy the β -lactam ring of the penicillins and cephalosporins, and those which inactivate chloramphenicol.

In clinical practice, resistance acquired by mutation is very important in tuberculosis. If a patient is put on streptomycin alone then initially organisms die in large numbers but soon resistant mutants appear which multiply in number while sensitive parents may be eliminated. If two or more drugs are used and if a mutant appears which is resistant to one of the drugs then it will be killed by other drug/s. **The chance of a mutant developing resistance to more than one drug at one time is remote.** This is the rationale of combined therapy in tuberculosis.

R plasmid-mediated resistance is usually to multiple drugs, therefore, there is no use of combined therapy. Table 7.1 shows important differences between mutational drug resistance and plasmid-mediated drug resistance. Acquisition of resistance by transduction is common in staphylococci. The penicillinase plasmids, which are transmitted by transduction, may also carry determinants of resistance to mercuric chloride and erythromycin.

Transposable genetic elements

Lederberg, in 1960, described an unusual class of *Gal*-mutants in *E. coli* caused by insertion of extra pieces of DNA called **insertion sequences** or **IS elements** at the point of mutation. Since then a large number of IS elements have been described in a wide variety of bacteria. They are common in bacterial plasmids and are common components of many, if not all, bacterial chromosomes. They are also **cryptic** in the sense that they do not confer on the host bacterium a predictable phenotype. Their presence is usually indicated by mutation. IS elements possess ability to transpose, i.e. they can insert into different sites on the same or on different DNA molecules.

In 1974, the discovery of a new type of transposable element that encoded a recognizable gene product (β -lactam antibiotic resistance) was reported. It was termed a **transposon (tn)**. Since then many others carrying a variety of resistance and other genes have been identified. Because of the ability of transposable elements to move from one plasmid to another or to a phage or to the bacterial chromosome they have assumed the popular name of **jumping genes**. **Unlike plasmids, transposons do not contain genetic information**

necessary for their own replication, and their replication, therefore, depends on their physical integration with a bacterial replicon.

Transposons are large (4–25 kb) transposable genetic elements. They encode at least one function that alters cell phenotype. Therefore, **transposon may be defined as a segment of DNA with one or more genes in the centre and the two ends carrying inverted repeat sequences of nucleotides—nucleotide sequences complementary to each other but in the reverse order**. Because of this feature each strand of the transposon can form a single-stranded loop carrying the gene/genes and a double-stranded stem formed by hydrogen bonding between the inverted repeat sequences (Fig. 7.8).

The transfer of genetic material from one DNA molecule to another is known as **transposition**. It does not need genetic homology between transposable element and its site of insertion. It is, therefore, different from recombination. Insertion of a transposon leads to the acquisition of new characteristics by the recipient DNA molecule. **It has been suggested that R plasmids may have evolved as collections of transposons each carrying a gene that confers resistance to one or more antibiotics.**

Genetic engineering (recombinant DNA technology)

Insertion of a foreign DNA molecule into DNA of a vector, which can replicate autonomously in a suitable host, is known as recombinant DNA (rDNA) technology or genetic engineering. This technique makes it possible to isolate any gene coding for any desired protein from microorganisms or from cells of higher forms of life including human beings, and their introduction into suitable microorganisms in which genes would be functional, directing the production of specific protein. Such cloning of genes in microorganisms enables the preparation of the desired protein in pure form, in large quantities and at a reasonable cost. It has been made possible by the discovery of DNA endonucleases or restriction endonucleases which are enzymes able to cut DNA molecules at specific sequences. These enzymes are present in many prokaryotic organisms, e.g. restriction endonucleases *EcoRI*, *HindIII*, *BamHI*, *PstI* and *SmaI* are obtained from

Table 7.1: Mutational and plasmid-mediated drug resistance

Mutational drug resistance	Plasmid-mediated drug resistance
- It is due to decreased permeability to drug, development of alternate metabolic pathways and development of enzymes inactivating the drug. - Involves resistance to one drug at one time. - Degree of resistance is usually low, therefore, infection may be treated by giving higher doses of the antibiotics. - Development of drug resistance can be prevented by treatment with combination of drugs. - Resistance is not transferable to other organisms. - Resistant mutants are usually metabolically defective. - Virulence of resistant mutants may be lowered.	- It is due to the development of degrading enzymes. - Involves many drugs at one time. - Degree of resistance is usually high, therefore, higher doses of antibiotics do not help. - Development of drug resistance cannot be prevented by treatment with combination of drugs. - Resistance is transferable to other organisms. - They are metabolically normal. - Virulence usually not decreased. It may rather be increased by acquisition of virulence plasmids.

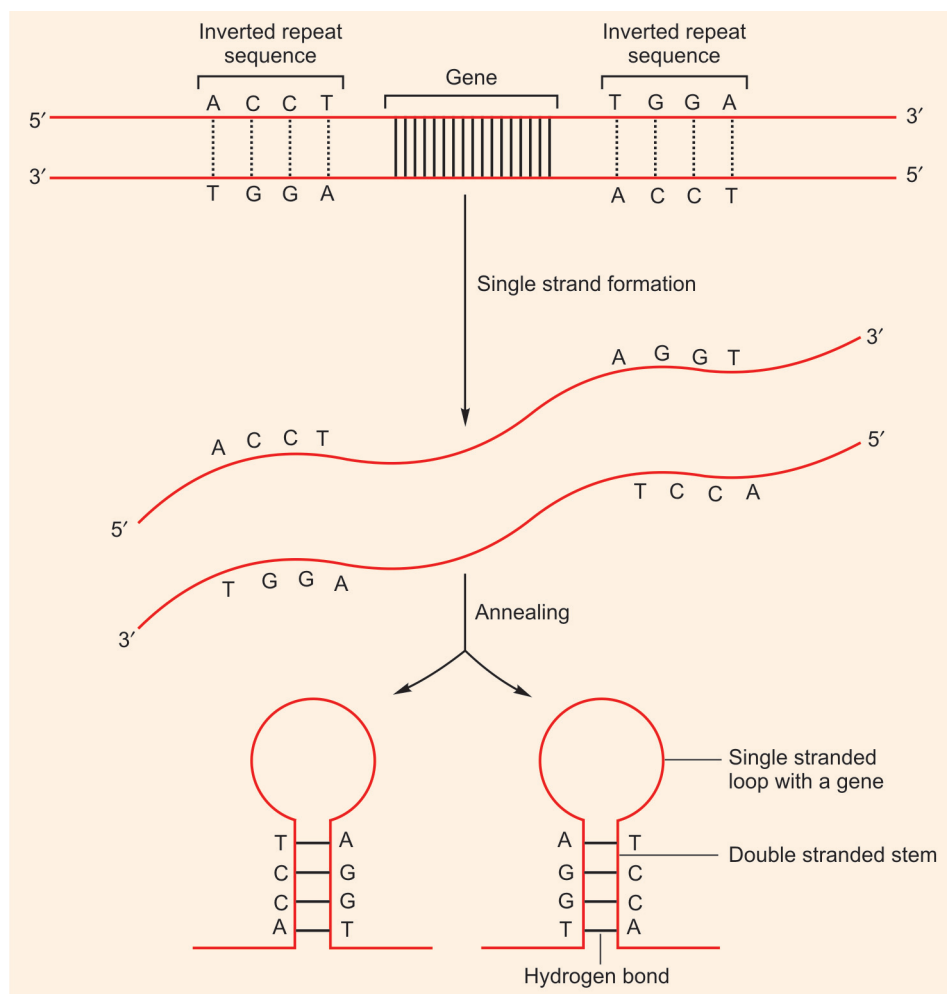
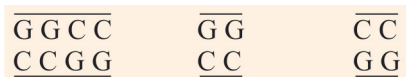


Fig. 7.8: Structure of transposon.

E. coli, *H. influenzae*, *Bacillus amyloliquefaciens*, *Providencia stuartii* and *Serratia marcescens*, respectively.

Restriction enzymes are produced by bacterial cells as part of their defence against the incursion of foreign DNA particularly bacteriophages. The target sites for these enzymes normally comprise four to six base pairs of specific sequence and are palindromic meaning that the sequence is the same on both strands of double helix. These enzymes cut double-stranded DNA in two different ways. Some cut at positions on the DNA strands opposite to each other giving blunt ends. Other enzymes cut off-centre but at same relative location in each strand generating complementary or cohesive or sticky ends that can overlap for two to four bases.



Cutting produces blunt ends.



Cutting produces complementary sticky ends.

Fragments of DNA cut with restriction enzymes can be rejoined by sealing or ligating enzyme known as DNA ligase.

The most commonly used ligase commercially available is that encoded by phage T4.

The **basic technique** is quite simple. Plasmid DNA from *E. coli* and chromosomal DNA from another organism are cleaved with restriction enzyme, mixed and released with DNA ligase. The recombinant DNA molecule is then introduced by transformation into *E. coli* where foreign chromosomal DNA can replicate because it is linked to a plasmid which has an origin of replication and the genes in the donor segment are said to be cloned and the DNA molecule that carries it is known as vector. Other vectors include λ phage of *E. coli* and simian virus 40. Plasmid vectors carrying genes that encode antibiotic resistance and other determinants are available. These facilitate selection of transformed cells that have acquired a plasmid that has incorporated a fragment of foreign DNA.

Applications of genetic engineering

- Genetic engineering is a useful tool for the **production of vaccines** (for prevention of disease) **and antigens** (for the diagnosis of disease).
- Yeast derived recombinant vaccine against hepatitis B is now commercially available.

- Genes coding for gp120 of human immunodeficiency virus (HIV) have been inserted into vaccinia virus. Inoculation of this hybrid-live virus (gp120/vaccinia hybrid vaccine) elicits both antibody-mediated and cell-mediated immunity against HIV.
- Vaccinia virus has also been used for the development of hybrid live vaccine against hepatitis B.
- Recombinant DNA technology has also been used for the **production of proteins** of therapeutic interest. These include human growth hormone, human insulin, erythropoietin, factor VIII, tissue plasminogen activator, interferons, tumour necrosis factor, interleukin-2, granulocyte colony stimulating factor, epidermal growth factor and fibroblast growth factor.
- An important application of recombinant technology is gene therapy. Normal genes can be introduced into the patient so that genetic diseases can be cured.

DNA probes

DNA probes are radiolabelled or chromogenically labelled pieces of single-stranded DNA which can be used for the detection of homologous DNA by hybridization.

Development of DNA probe

All microorganisms, simple or complex, contain some unique sequences of nucleic acid within their genome that distinguish them from all other organisms. The method of developing a DNA probe is to cut or isolate those sequences from the nucleic acid of the cell using a set of enzymes known as restriction endonucleases, reproduce them in large quantities and attach a reporter molecule to them so that they can be incorporated into a hybridization reaction. **Hybridization** is the process whereby two single strands of nucleic acid come together to form a stable double-stranded molecule. However, they will bind and stay together only if the sequences of bases along each stretch of nucleic acid are complementary (adenine opposite thymine and cytosine opposite guanine).

Hybridizations are accomplished in tubes or by spotting the unknown organisms on a filter paper such as nitrocellulose paper, lysing them to release the DNA, and denaturing in mild alkali to single strands. The probe can then be added and after thorough washing to remove any unbound probe, the tube or probe is examined for evidence of hybridization. Formalin-fixed, paraffin-embedded tissues can also be probed particularly for viral DNA sequences. DNA probes can also be designed that bind to RNA and this procedure has been used particularly to locate ribosomal RNA.

Applications of DNA probes

- In the diagnostic laboratory, DNA probes are being used for culture confirmation as an alternative to conventional, time-consuming or labour-intensive methods. For example, DNA hybridization makes it possible to rapidly identify *Mycobacterium tuberculosis*, *M. kansasii*, *M. avium* complex, and *M. gordonae* isolated in culture, significantly reducing the time for reporting of the species of the isolate.

- Probe technology may also be used for detection of fastidious organisms directly in clinical specimens. Examples are *Neisseria gonorrhoeae* and *Chlamydia trachomatis*.

Blotting techniques

Southern blotting

DNA fragments are obtained by restriction enzyme digestion and are separated by gel electrophoresis. These fragments are then transferred from the gel to nitrocellulose or nylon membranes that bind DNA. The DNA bound to the membrane is denatured (converted to single-stranded form) and then hybridized with radioactive single-stranded DNA probes. This results in the formation of radioactive double-stranded segments which can be detected on X-ray film. This is a highly sensitive technique for identifying DNA fragments by DNA:DNA hybridization. This is known as Southern blotting after EM Southern who developed it.

Northern blotting

A similar procedure for analysis of RNA has been called northern blotting. Here, the RNA is separated by gel electrophoresis, blotted and identified by using radiolabelled probes.

Western blotting

See Chapters 13 and 63.

Polymerase chain reaction (PCR)

Kary Mulis invented this method in 1989. He was awarded Nobel Prize in 1993. Polymerase chain reaction is a primer-mediated, temperature-dependent technique for the enzymatic amplification of a specific sequence (target sequence) to such an extent that it can be detected. The technique can be used to detect very small amounts of specific nucleic acid material in clinical specimens where bacterial, viral or fungal agents are thought to play a causative role. The fundamental basis of this technology is that each pathogenic organism possesses a unique 'signature sequence' in its DNA or RNA composition by which it can be identified. It is based on repeated cycles of high temperature template denaturation, oligonucleotide primer annealing, and polymerase mediated extension (Fig. 7.9).

To multiply a strip of genetic material, four ingredients are placed together in a small vial:

1. Target DNA.
2. Short strands of DNA called primers which tag the section to be copied.
3. Polymerase—an enzyme that promotes gene replication in all living cells.
4. Nucleotides—the building blocks for making DNA.

PCR is carried out in three steps:

1. Heat at 94°C is applied to the target DNA, breaking the bonds that hold the strands together. This is known as **denaturation**.
2. The temperature is then reduced to 55°C, promoting the primers to attach themselves to either end of the target strip. This is known as **annealing of primers**.

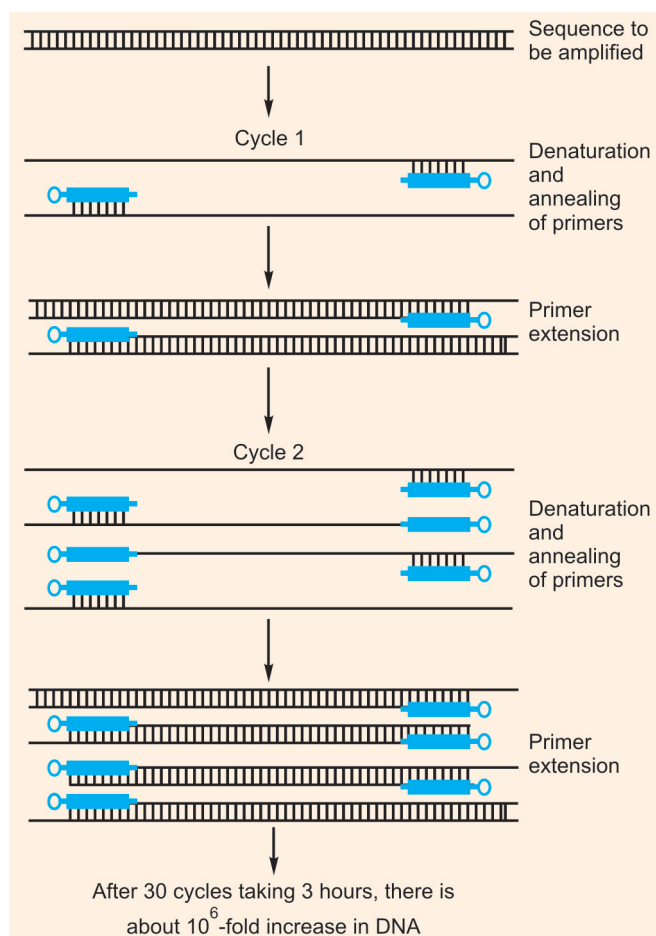


Fig. 7.9: Polymerase chain reaction.

- Then polymerase enzyme triggers the formation of new DNA strand from the nucleotides. Extension of the primers is done by a thermostable *Taq* polymerase (purified from *Thermus aquaticus*, a thermophilic bacterium that lives in hot springs at temperatures of 70–75°C). This is known as **primer extension**. When the temperature is again raised, the new strands separate and the process begins again.

These three steps are repeated again and again by manipulating the temperature, a process that is automated by the PCR machine (Table 7.2). A cycle takes about 3–5 minutes and after 30 cycles, taking about 3 hours, a single copy of DNA can be increased up to 1,000,000 copies, a

Table 7.2: Programming of cycles in polymerase chain reaction

Step	Temperature	Time	Cycle
Initial denaturation	94°C	5 minutes	First cycle
Denaturation	94°C	30 seconds	30 cycles
Annealing of primers	55°C	1 minute	
Primer extension	72°C	1 minute	
Final primer extension	72°C	7 minutes	Last cycle

sharp contrast to the days required by conventional amplification method (culture).

Amplified sequences of target DNA can be detected by a variety of methods. If enough amplified DNA is present, it can be visualized by:

1. Gel electrophoresis and ethidium bromide staining.
2. Southern blot and dot-blot analysis with either radioactive or non-radioactive probes.
3. Oligomer restriction.
4. Oligomer hybridization.
5. Reverse dot-blot.

Applications of PCR

The development of PCR or gene amplification method is a major methodological breakthrough in molecular biology. Within a short span, this method has found its way into nearly every type of laboratory from forensic to ecology and from diagnosis to pure research. Applications of PCR in clinical laboratory are given in Table 7.3.

Table 7.3: Applications of PCR in clinical laboratory

Diagnosis of infections due to:

- Viruses:** HIV-1, HIV-2, HTLV-1, cytomegalovirus, human papillomavirus, herpes simplex viruses, hepatitis B virus, HCV, HDV, HEV, rubella virus, Epstein-Barr virus, varicella-zoster virus, human herpesvirus 6 and 7, *Parvovirus* B19, enteroviruses, coxsackieviruses, echoviruses, rhinoviruses, measles virus, rotavirus, adenovirus, respiratory syncytial virus
- Bacteria:** *Mycobacterium tuberculosis*, *Mycobacterium avium* complex, *Legionella pneumophila*, *Chlamydia trachomatis*, *Mycoplasma pneumoniae*, *Helicobacter pylori*, *Burkholderia pseudomallei*, *Campylobacter* spp., *Corynebacterium diphtheriae*, *Leptospira interrogans*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Yersinia enterocolitica*
- Fungi:** *Candida* spp., *Cryptococcus neoformans*, *Aspergillus* spp., *Pneumocystis jirovecii*
- Protozoa:** *Toxoplasma gondii*, *Trypanosoma cruzi*, *Entero-cytozoon bieneusi*, *Encephalitozoon hellem*, *Plasmodium* spp.

- In diagnosis of inherited disorders:** The PCR technology is being widely used to amplify gene segments that contain known mutations for diagnosis of inherited diseases such as sickle cell anaemia, β -thalassaemia, cystic fibrosis, etc. PCR is especially useful for prenatal diagnosis of inherited diseases, where cells obtained from the foetus by amniocentesis are very few.
- In cancer detection:** Identification of mutations in onco-suppressor genes such as retinoblastoma gene can help to identify individuals at high risk of cancer.
- In medicolegal cases:** PCR allows DNA in a single cell, hair follicle or sperm to be amplified enormously and analyzed. The pattern obtained is then compared with that of various suspects.

Other types of PCR

Other types of PCR include reverse transcriptase PCR (RT-PCR), nested PCR, multiplex PCR and real time PCR.

RT-PCR

In this technique target is RNA instead of DNA. Complementary DNA (cDNA) is first produced from RNA with the help of enzyme reverse transcriptase and then cDNA is amplified by PCR.

Nested PCR

This increases the sensitivity and specificity of PCR by using two pairs of amplification primers. One pair of primers is used in the first round of PCR to amplify the desired sequence. The amplified product of the first round is then subjected to second round of PCR with the second set of primers which anneal to the sequences of the first round products.

Multiplex PCR

In this technique two or more primer sets are used for amplification of different targets. It helps in amplification of more than one target sequence in a clinical specimen.

Real time PCR

In this technique amplified products are detected as they accumulate after each cycle, in contrast to standard PCR where these are detected at the end of procedure. Therefore, a positive result can be obtained, while the assay is still running. It uses a fluorescent reporter dye. Real time PCR instrument has the ability to measure the increase in reporter fluorescence as PCR products accumulate.

KEY POINTS

- **DNA** molecule is composed of two chains of nucleotides wound together in the form of double helix.
- **DNA replication** is the synthesis of new strands of DNA using original DNA strands as templates; DNA-dependent DNA polymerase is the main enzyme that mediates DNA replication.
- **Genetic variation** in bacteria can occur either by **mutation** or **gene transfer**.
- **Mutation** is a random, undirected, heritable variation caused by an alteration in the nucleotide sequence at some point of the DNA of the cell, which may be due to **addition, deletion or substitution of one or more bases**.
- **Gene transfer** in bacteria may occur by **conjugation, transduction, transformation or transposition**.
- **Plasmids** are **extrachromosomal, double-stranded circular DNA molecules** capable of **independent replication** within the bacterial host.
- The **clinical relevance** of plasmids lies in the fact that they code for antibiotic resistance, resistance to heavy metals, exotoxin production and sex pilus formation.
- **Transposons** are '**jumping genes**' that move from one site to another either within or between the DNA molecules.
- DNA probes used in **diagnostic microbiology** are labelled (with chemicals or radioactive phosphate groups) pieces of DNA that can be used to detect specific sequences of DNA of the pathogens (in the clinical sample) by pairing with the complementary bases.
- The **polymerase chain reaction** is a widely used technique that enables multiple copies of a DNA molecule to be generated by enzymatic amplification of target DNA sequence.

Important Questions

1. What is the genetic code? Give the properties of the genetic code.
2. What is genetics? Describe the structure and functions of DNA and RNA.
3. Describe the structure and functions of the plasmids.
4. Describe lac operon.
5. Define mutation. Discuss various types of mutations.
6. Write short notes on
 - (a) Transformation
 - (b) Transduction
 - (c) Conjugation
 - (d) F factor
 - (e) R plasmid
7. Differentiate between mutational and plasmid-mediated drug resistance in a tabulated form.
8. Write short notes on:
 - (a) Transposable genetic elements
 - (b) Recombinant DNA technology
9. What are DNA probes? Discuss the development of nucleic acid probes and their clinical applications.
10. Discuss polymerase chain reaction. What are the applications of this reaction in clinical practice?



Multiple Choice Questions

- Which of the following is nonsense codon?
(a) UAA. (b) GGC.
(c) AAG. (d) CGA.
- Plasmids which **do not possess** information for self-transfer to another cell are known as:
(a) conjugative plasmids.
(b) cryptic plasmids.
(c) non-conjugative plasmids.
(d) F plasmids.
- Which of the following properties may be plasmid-mediated?
(a) Resistance to antibiotics.
(b) Production of enterotoxin.
(c) Fermentation of lactose.
(d) All of the above.
- Lactose fermentation requires which of the following enzymes?
(a) β -galactosidase. (b) Galactoside permease.
(c) Transacetylase. (d) All of the above.
- Genotypic variations are:
(a) stable.
(b) not heritable.
(c) temporary.
(d) influenced by the environment.
- Acquisition of naked DNA by a bacterium from its environment and incorporation in its genome is known as:
(a) transformation.
(b) transduction.
(c) lysogenic conversion.
(d) conjugation.
- Plasmids which possess both resistance transfer factor (RTF) and resistance (*r*) determinants are known as:
(a) conjugative plasmids.
(b) non-conjugative plasmids.
(c) non-self-transmissible plasmids.
(d) F plasmids.
- Drug resistance in tuberculosis is due to:
(a) mutation. (b) conjugation.
(c) transduction. (d) transformation.
- Acquisition of penicillin resistance in staphylococci is due to:
(a) mutation. (b) conjugation.
(c) transduction. (d) transformation.
- A segment of DNA with one or more genes in the centre and the two ends carrying inverted repeat sequences of nucleotides is known as:
(a) insertion sequence.
(b) transposon.
(c) plasmid.
(d) none of the above.
- Diagnostic DNA probes have been developed for:
(a) hepatitis B virus.
(b) human immunodeficiency virus.
(c) *Mycobacterium tuberculosis*.
(d) all of the above.
- Transfer of genetic information from DNA to RNA is called:
(a) transduction.
(b) transcription.
(c) transformation.
(d) recombination.
- Transmission of a piece of DNA from one bacterium to another by a bacteriophage is known as:
(a) transduction.
(b) transformation.
(c) conjugation.
(d) translation.
- Which of the following are referred to as 'jumping genes'?
(a) Plasmids.
(b) Transposons.
(c) Bacteriophages.
(d) Operons.
- Transfer of genetic material through direct contact between donor and recipient bacteria is called:
(a) transformation.
(b) conjugation.
(c) transduction.
(d) sexduction.
- When a temperate phage incorporates its DNA into the host cell's genome, it is called a:
(a) lysophage.
(b) lytic phage.
(c) prophage.
(d) coliphage.



Answers

- | | | | | | | | | | | | |
|-------|-------|-------|-------|------|------|------|------|------|-------|-------|-------|
| 1. a | 2. c | 3. d | 4. d | 5. a | 6. a | 7. a | 8. a | 9. c | 10. b | 11. d | 12. b |
| 13. a | 14. b | 15. b | 16. c | | | | | | | | |

Bacteria in Health and Disease

Competency achievement: The student after reading the chapter should be able to:

MI1.1: Describe different causative agents of infectious diseases, the methods used in their detection, and discuss the role of microbes in health and disease.

MI8.1: Enumerate the microbial agents and their vectors causing zoonotic diseases. Describe the morphology, mode of transmission, pathogenesis and discuss the clinical course, laboratory diagnosis and prevention.

CM3.6: Describe the role of vectors in the causation of diseases.

Skin, alimentary tract and other mucous membranes are continually contaminated by microorganisms from the environment. Based on their relationship they can be divided into saprophytes and parasites.

Saprophytes

Saprophytes (*sapros*—decayed, and *phyton*—plant) are free-living microbes that live on dead or decaying organic matter. They are found in soil and water. They are, generally, incapable of multiplying on living tissues. However, sometimes when host resistance is lowered some saprophytes like *Bacillus subtilis* may cause infection.

Parasites

Parasites are the microorganisms that can enter and multiply in the host. They are of two types—**microparasites** which include viruses, bacteria, fungi and protozoa, and **macroparasites** which include helminths.

Pathogens

Pathogens (*pathos*—suffering, and *gen*—produce, i.e. disease-producing) are the microorganisms which are capable of producing disease in man and animals. They are of two types—opportunistic pathogens and primary pathogens.

Opportunistic pathogens

Opportunistic pathogens rarely cause disease in individuals with intact immunological and anatomical defences. In immunocompromised hosts these bacteria are able to cause

disease. Coagulase-negative staphylococci are normally carried on the human skin where they cause no harm. However, introduction of these organisms into anatomical sites in which they are not normally found may lead to infection. Similarly, *Escherichia coli* is normally carried in human intestine. If it enters into urinary tract it leads to urinary tract infection.

Primary pathogens

Primary pathogens are the organisms which are capable of causing disease in previously healthy individuals with intact immunity.

Commensals

Commensals (organisms of normal flora) are the microorganisms which live in complete harmony with the host without causing any damage to it. They constitute normal flora of the body.

The organisms of normal flora differ at different parts of the body (Table 8.1). This is due to local environmental factors which tend to select certain species. These organisms obtain their nutrition from the secretions and waste products of the body.

Microorganisms that colonize an area for months or years represent **resident microbial flora**, whereas microorganisms that are present at a site temporarily represent **transit flora**. Transit flora comes to 'visit' but does not usually live or stay. These organisms are eliminated either by the host inherent immune defenses or by competition with the resident flora.

INFECTION

The lodgement and multiplication of a parasite in or on the tissue of a host is known as infection.

Types of infection

1. Primary infection

Initial infection with a parasite is known as primary infection.

2. Reinfection

Subsequent infection with the same parasite in the same host is known as reinfection.

Table 8.1: Normal flora at various sites of the body

Site	Organisms
1. Skin	<i>Staphylococcus epidermidis</i> , <i>S. aureus</i> , <i>Micrococcus</i> spp., <i>Candida</i> spp. <i>Clostridium</i> spp., diphtheroids, α -haemolytic and non-haemolytic streptococci, <i>Acinetobacter</i> spp., <i>Bacteroides</i> spp., Gram-negative rods (fermenters and non-fermenters), <i>Moraxella</i> spp.
2. Nares and nasopharynx	<i>S. aureus</i> , <i>S. epidermidis</i> , <i>Corynebacterium</i> spp., <i>Peptostreptococcus</i> spp., <i>Fusobacterium</i> spp., <i>Streptococcus</i> spp., <i>Neisseria</i> spp. including <i>N. meningitidis</i> , <i>S. pneumoniae</i> , <i>Prevotella</i> spp., <i>Haemophilus</i> spp., yeasts.
3. Mouth	<i>S. epidermidis</i> , <i>S. aureus</i> , <i>S. mitis</i> , <i>S. sanguis</i> , <i>S. salivarius</i> , <i>S. mutans</i> , <i>Peptostreptococcus</i> spp., <i>Veillonella</i> spp., <i>Actinomyces israelii</i> , <i>Bacteroides</i> spp., <i>Treponema denticola</i> , <i>T. refringens</i> , <i>Neisseria</i> spp., lactobacilli, <i>Candida</i> spp., <i>Entamoeba gingivalis</i> , <i>Trichomonas tenax</i> .
4. Oropharynx	<i>S. aureus</i> , <i>S. epidermidis</i> , <i>S. pneumoniae</i> , <i>S. mitis</i> , <i>S. mutans</i> , <i>S. milleri</i> , <i>S. sanguis</i> , <i>S. salivarius</i> , <i>Moraxella catarrhalis</i> , <i>H. parainfluenzae</i> , <i>H. influenzae</i> , anaerobic streptococci, <i>Bacteroides</i> spp., <i>Prevotella</i> spp., <i>Porphyromonas</i> spp., <i>Fusobacterium necrophorum</i> , <i>S. pyogenes</i> , <i>N. meningitidis</i> .
5. Gastrointestinal tract	<i>Bacteroides</i> spp., <i>Clostridium</i> spp., Enterobacteriaceae, <i>Eubacterium</i> spp., <i>Fusobacterium</i> spp., <i>Enterococcus</i> spp., <i>Peptostreptococcus</i> spp., <i>Peptococcus</i> spp., <i>S. aureus</i> , <i>Lactobacillus</i> spp., yeasts.
6. Genitourinary tract	<i>Lactobacillus</i> spp., <i>Bacteroides</i> spp., <i>Clostridium</i> spp., <i>Peptostreptococcus</i> spp., <i>S. aureus</i> , <i>S. epidermidis</i> , <i>Enterococcus</i> spp., diphtheroids, group B streptococci, Enterobacteriaceae, <i>Acinetobacter</i> spp., <i>Candida albicans</i> .
7. Conjunctiva	<i>Corynebacterium</i> spp., <i>S. epidermidis</i> , non-haemolytic streptococci, neisseriae.

3. Secondary infection

When the primary infection lowers the resistance of the host and the latter gets infection with another organism it is known as secondary infection.

4. Cross infection

When a patient already suffering from a disease acquires a new infection it is known as cross infection.

5. Nosocomial infection

When cross infection is acquired by the patient during his stay in the hospital it is known as hospital-acquired, healthcare-associated or nosocomial infection.

6. Iatrogenic infection

When the infection is acquired during therapeutic or investigative procedures, it is known as iatrogenic or physician-induced infection.

7. Subclinical infection

When the clinical symptoms of an infection are not apparent it is known as subclinical infection.

8. Latent infection

When a parasite, after infection, remains in a latent or hidden form for some time and it proliferates and produces clinical disease when the host resistance is lowered, it is known as latent infection.

Sources of infection

Infections may be endogenous, due to the organisms of the normal flora, and exogenous, due to the organisms derived from a source outside the body.

Endogenous infections

Organisms of normal flora are usually non-pathogenic but occasionally they may lead to infection. The most important endogenous infections are:

1. **Viridans streptococci** are the normal flora of the mouth but when there is abnormality of the heart, like rheumatic heart disease and injury to the oral cavity like tooth extraction or fractured mandible, these organisms enter into the blood stream and settle down on the heart leading to infective endocarditis. In both the above examples, two conditions are fulfilled—the organism initiating infection does so in an area of the body remote from its normal habitat and infection develops only where there is some tissue abnormality lowering local tissue resistance.
2. ***E. coli* and *Enterococcus faecalis***, which are the normal flora of the intestines, may cause urinary tract infection particularly when there is some abnormality of the urinary tract like congenital malformation, urinary calculi and ureteric and prostatic obstruction. These conditions lower the local tissue resistance.

Exogenous infections

Most of the infections are exogenous in origin. The sources of exogenous infections are as under:

1. Human cases and carriers.
2. Animal cases and carriers.
3. Insects.
4. Environment.

1. Human cases and carriers

The commonest source of human infection is man himself who may be a patient or a carrier. Infections due to some organisms are acquired mainly or exclusively from ill persons, e.g. AIDS, syphilis, gonorrhoea, pulmonary tuberculosis, leprosy, whooping cough, hepatitis B and C, measles, mumps, influenza, poliomyelitis, etc.

A **carrier** is a person who harbours the pathogenic microorganisms without suffering from it. There are several types of carriers:

- **Healthy carrier:** One who harbours the pathogen but has never suffered from the disease caused by it.

- *Convalescent carrier*: One who has recovered from the disease but continues to harbour the pathogen on his body.
- *Temporary carrier*: When carrier state lasts for less than 6 months.
- *Chronic carrier*: When carrier state lasts for years or may be for the life of the patient.
- *Paradoxical carrier*: Who acquires the organisms from another carrier.
- *Contact carrier*: Who acquires the organisms from a patient.

Carriers are very important source of infection. For example, a person acquires organisms from a patient of meningococcal meningitis and becomes a contact carrier. He is then a source of infection for other persons (patient → carrier → patient).

2. Animal cases and carriers

Certain pathogens are capable of causing infection in both man and animals. Therefore, animals may act as a source of infection of such organisms. The infection may be acquired by contact with the animal, animal bite and ingestion of milk or meat. Infection in animals may be asymptomatic and these animals may serve as reservoir for human infections. These are known as **reservoir hosts**. Infectious diseases transmitted from animals-to-man are known as **zoonoses**. For example:

- *Bacterial*: Bovine tuberculosis, bubonic plague, *Salmonella* food poisoning, anthrax.
- *Viral*: Rabies, yellow fever.
- *Protozoal*: Leishmaniasis.
- *Helminthic*: Hydatid disease.
- *Fungal*: *Microsporum canis*, *Trichophyton verrucosum*.

3. Insects

Blood-sucking insects such as mosquitoes, ticks, mites, flies, and lice act as a source of a number of human and animal infections. Insects transmitting pathogens are known as **vectors**.

4. Environment

This includes soil, water and food. A few infective diseases of man are caused by saprophytic microbes derived from soil and vegetation. Some pathogens can survive in the environment for very long periods. For example, spores of tetanus and gas gangrene bacilli remain viable in the soil for several decades and serve as a source of infection. The normal habitat of these organisms is the human and animal intestines and they enter the soil through their faeces.

Fungi causing mycetoma, sporotrichosis and histoplasmosis survive in soil and cause human infection. Eggs of parasites like those of roundworms and hookworms survive and develop in the soil and cause human infection. Water contaminated with *Shigella*, *Salmonella*, *Vibrio cholerae*, poliovirus, hepatitis A and E viruses and cyclops containing larvae of guinea worm acts as a source of these infections.

Contaminated food acts as a source of organisms causing food poisoning, gastroenteritis, diarrhoea and dysentery.

Modes of spread of infection

Pathogenic organisms can spread from one host to another by a variety of mechanisms. These include:

1. Inhalation

Respiratory infections such as common cold, influenza, measles, mumps, tuberculosis and whooping cough are acquired by inhalation. These organisms are shed into the environment by patients in secretions of nose or throat during sneezing, coughing, talking and other forceful expiratory activities. These activities expel a spray of droplets. **Large droplets** more than 0.1 mm in diameter fly forwards and downwards from the mouth to the distance of a few feet and they reach the floor within a few seconds or they may fall on the eyes, face, mouth and clothes of the person standing in front of the producer of the spray. Small droplets, less than 0.1 mm in diameter, evaporate immediately to become minute residue or **droplet nuclei** and remain airborne and may be inhaled into the nose, throat or lungs.

2. Ingestion

Intestinal infections like enteric fever, cholera, dysentery, food poisoning, poliomyelitis, hepatitis A and E, and most of the parasitic infections are acquired by ingestion. The source of these infections is the faeces of the patients or carriers. The faeces containing pathogens may contaminate food and drinks.

3. Contact

Infection may be acquired by direct or indirect contact with the patient. Sexually transmitted diseases (STD) such as syphilis, gonorrhoea, lymphogranuloma venereum, lymphogranuloma inguinale, trichomoniasis, herpes simplex, hepatitis B and C, and AIDS are acquired by direct contact. The term **contagious disease** is used for the disease acquired by direct contact, and disease acquired by other modes, through inanimate objects, as **infectious disease**.

4. Contamination of wounds

The infections may be caused by:

- Organisms present in the nose or throat of the patient himself or of nurses or doctors. Pathogenic staphylococci and streptococci derived from respiratory tract are important causes of wound and burn infection.
- Airborne spread of organisms from the infected wounds of other patients.
- Contact with infected hands, clothing or other articles.
- In some instances pathogens may be inoculated directly into the tissues of the host, for example, rabies virus which is present in the saliva of a rabid animal, usually dog, is inoculated directly into the host tissue.
- Spores of *Clostridium tetani* and *C. perfringens* are present in the soil. These get inoculated into the host tissue following severe wounds leading to tetanus and gas gangrene, respectively.

5. Blood-sucking arthropods

In some diseases, blood-sucking insects play an important role in the spread of infection from one individual to another. Table 8.2 shows common arthropods and diseases transmitted by them.

Table 8.2: Common arthropods and diseases transmitted by them

Arthropod	Diseases transmitted
1. Mosquitoes	
• <i>Anopheles</i>	Malaria, filariasis
• <i>Culex</i>	Filariasis, Japanese encephalitis, West Nile fever
• <i>Aedes</i>	Yellow fever, dengue, chikungunya haemorrhagic fever, Rift Valley fever, filariasis
• <i>Mansonia</i>	Brugian filariasis
2. Flies	
• Housefly	Typhoid and paratyphoid fever, diarrhoea, dysentery, cholera, gastro-enteritis, amoebiasis
• Sandfly	Kala-azar, oriental sore, espundia, oroya fever
• Tsetse fly	African trypanosomiasis
3. Louse	Epidemic typhus, relapsing fever, trench fever
4. Rat flea	Bubonic plague, endemic typhus, <i>Hymenolepis diminuta</i> infection
5. Black fly	Onchocerciasis
6. Reduviid bug	Chagas' disease
7. Ticks	
• Hard ticks	Spotted fever group, viral encephalitis, Colorado tick typhus, tularaemia, human babesiosis
• Soft ticks	Q fever, relapsing fever, Kyasanur Forest disease
8. Mites	
• Trombiculid mite	Scrub typhus
• Gamasid mite	Rickettsial pox
• Itch mite	Scabies
9. Body and head lice	Epidemic typhus, relapsing fever
10. Cyclops	Dracunculosis, diphyllbothriasis
11. Cockroaches	Same as in case of housefly

Insects normally become infected by biting a human or animal host in whose blood the causative organism is present. After this there is an interval, known as **extrinsic incubation period**, during which the insect is incapable of transmitting the infection. During this period the organisms multiply in the body of the insect. *Ticks which transmit certain rickettsial and arbovirus infections, and mites which transmit scrub typhus are unusual in that the infective agent can be transmitted from one generation of the insect to the next through the ovum.*

Arthropods transmit infection in four ways:

1. The infective agent gains access to the salivary glands of the insect and the organisms enter into the wound caused by the insect bite along with the saliva, e.g. transmission of malaria by female *Anopheles* mosquito.
2. The infective agent multiplies in the stomach of the insect and blocks the proventriculus, and during feeding it is regurgitated into the wound, e.g. transmission of bubonic plague by rat flea.
3. The agent multiplies in the intestinal tract and is excreted in the faeces. These are deposited by the insect besides the wound when it bites and due to irritation caused by

insect bite, are scratched by the victim into the wound, e.g. transmission of rickettsial diseases.

4. The infective agent multiplies in the coelomic cavity of the insect and infection is due to contamination of the wound of bite with the coelomic fluid of an insect that has been crushed in its vicinity by the victim, e.g. transmission of louse-borne relapsing fever.

6. Iatrogenic and laboratory-acquired infections

If meticulous care in asepsis is not taken, infections like AIDS, and hepatitis B, C and D may sometimes be transmitted during therapeutic and investigative procedures. These are known as iatrogenic infections. Laboratory personnel handling infectious material are particularly at risk.

7. Congenital

Some microorganisms like *Toxoplasma*, rubella virus, cytomegalovirus, herpes simplex virus, varicella-zoster virus, human immunodeficiency virus, *Treponema pallidum*, malaria parasites, etc. can cross the placental barrier and infect the foetus *in utero*. This is known as **vertical transmission**. This may result in abortion, miscarriage or stillbirth. Live babies may be born with manifestations of the disease.

Another form of transmission from mother to infant occurs by contact during birth with organisms such as group B streptococci, *Chlamydia trachomatis* and *Neisseria gonorrhoeae* which colonize the vagina. Herpes simplex virus, cytomegalovirus and human immunodeficiency virus may be present in the genital secretions and baby may acquire these during birth. Cytomegalovirus and human immunodeficiency virus may be transmitted by breast milk, a third mechanism of vertical transmission.

Factors predisposing to microbial pathogenicity

Pathogenicity denotes the ability of a microbial species to cause disease, while the term **virulence** refers to the same property in a strain of the species.

Enhancement of virulence of a strain is termed as **exaltation**. This can be induced by serial passage of a strain in an experimental animal. Reduction of virulence of a strain is termed **attenuation**. This can be induced by repeated passage through unfavourable hosts and repeated culture in artificial media.

Determinants of virulence

1. Adhesion

Many bacteria possess on their surface colonization factors or adhesins. These usually occur on fimbriae. Through adhesins bacteria attach specifically on the receptors present on the host cells. They are, therefore, responsible for tissue tropism. Adhesion is necessary to avoid innate host defence mechanisms such as peristalsis in the gut and the flushing action of mucus, saliva and urine which remove non-adherent bacteria. Loss of adhesins may render a strain avirulent.

2. Invasion of tissues

Invasiveness signifies the ability of an organism to penetrate a tissue after it adheres to a cell surface. Some bacteria can invade tissues in the absence of physical injury, e.g.

N. meningitidis in nasal epithelium and salmonellae in intestinal epithelium. These organisms are endocytosed by epithelial cells, transported across these cells within vacuoles and released into the submucosal space, from which they invade the underlying tissues. *Shigella* and enteroinvasive *E. coli* are also endocytosed by intestinal epithelial cells but do not penetrate the basement membrane.

3. Capsules

Cell wall in many bacteria is enclosed by a protective gelatinous covering layer known as capsule. It contributes to the virulence of the bacteria by inhibiting phagocytosis. Virtually all the pathogens associated with meningitis and pneumonia including *H. influenzae*, *N. meningitidis*, and *S. pneumoniae* are capsulated, and non-capsulated variants usually exhibit much reduced virulence. Some bacterial surface antigens such as Vi antigen of *S. serotype Typhi* and K antigen of *E. coli* also help the bacteria to withstand phagocytosis and the lytic activity of complement.

4. Streptococcal M protein

The M protein present on the surface of *S. pyogenes* binds both fibrinogen and fibrin to the bacterial cell wall thus masking the bacterial receptors from complement.

5. Bacterial toxins

These are substances produced by or present in bacteria, which have a direct toxic action on tissue cells. Two major types of toxin have been described—endotoxins and exotoxins (Table 8.3).

Endotoxins

They are components of the outer membrane of Gram-negative bacteria. They are lipopolysaccharide (LPS) in nature and are released from the bacterial surface by natural lysis of the bacteria or by disintegration of the organisms *in vitro*. The endotoxic activity of LPS resides in its lipid A moiety. The latter is not destroyed by autoclaving, hence infusion of a sterile solution containing endotoxin can cause

serious illness. They are poor antigens and the toxicity is not completely neutralized by the homologous antibodies. They cannot be toxoided. Endotoxins exert a wide spectrum of effects on the host, the most dramatic of which are fever and the shock syndrome associated with Gram-negative bacterial sepsis. Man is particularly sensitive to minute amounts of endotoxins and often a mild Gram-negative bacterial infection will cause fever. Larger amounts of endotoxin may cause irreversible shock seen in association with a fulminating Gram-negative bacteraemia.

Exotoxins

They are produced extracellularly by both Gram-positive and Gram-negative bacteria. They are highly potent even in small amounts and constitute some of the most poisonous substances known. **Botulinum toxin is the most poisonous followed by tetanus toxin.** Minimum lethal dose for botulinum toxin for a mouse is 0.03 ng and for humans may be 1 µg. It has been estimated that 3 kg of this toxin can kill all the inhabitants of the world. Exotoxins are heat-labile, protein in nature and have enzymatic activity. Some exotoxins can be partially denatured by treatment with formaldehyde to generate toxoids which lack toxicity but retain antigenicity inducing protective immunity when used as vaccines. Some exotoxins like diphtheria toxin, and enterotoxins of cholera vibrio and *E. coli* consist of two fragments A and B. The toxin binds to the specific receptors, on the host cell surface, through fragment B (binding) and then toxic or enzymatic A fragment causes cell damage.

6. Resistance to killing by phagocytic cells

Some pathogens like tubercle bacilli can be readily ingested (phagocytosed) by macrophages and other phagocytes but they resist intracellular killing by preventing fusion of phagosome with lysosome. These organisms rather multiply inside these cells. Other bacteria such as *S. aureus* and *N. gonorrhoeae* are able to resist the action of lysosomal components following fusion.

Table 8.3: Differences between exotoxins and endotoxins

Exotoxins	Endotoxins
- Proteins with high molecular weight ranging from 10,000 to 900,000. - Heat-labile. The toxicity is destroyed by heating above 60°C. - Highly antigenic; stimulate formation of antitoxin which neutralizes toxin. - Actively secreted by the cells; diffuse into the surrounding medium. - Converted into toxoid by formaldehyde. - Action often enzymic. - Specific pharmacological effect for each exotoxin. - Highly specific for particular tissue, e.g. tetanus toxin for CNS. - Very high potency (1 mg of botulinum or tetanus toxin can kill more than 1 million guinea pigs). - Do not produce fever in the host. - Produced by both Gram-positive bacteria and Gram-negative bacteria. - Frequently controlled by extrachromosomal genes (e.g. plasmids).	- Lipopolysaccharide in nature. Lipid A portion is probably responsible for the toxicity. - Heat-stable; can withstand heat over 60°C without losing toxicity. - Weakly antigenic; do not stimulate the formation of antitoxin. - Antibodies against only polysaccharide component are raised. - Form integral part of the cell wall; do not diffuse into surrounding medium. These can be obtained only by cell lysis. - Cannot be toxoided. - No enzymic action. - Non-specific action of all endotoxins. - Non-specific in action. - Low potency (1 mg of extracted somatic antigen can kill 1 mouse). - Usually produce fever in the host. - Produced by Gram-negative bacteria only. - Synthesis directed by chromosomal genes.

KEY POINTS

- **Pathogens** are microorganisms which are capable of producing disease in man.
- **Commensals** (organisms of normal flora) are the microorganisms which live in complete harmony with the host without causing any damage to it.
- Lodgement and multiplication of a parasite in or on the tissue of a host is known as **infection**.
- Infection may be **endogenous**, due to the organisms of the normal flora, and **exogenous**, due to the organisms derived from a source outside the body.
- Sources of exogenous infections are human cases and carriers, animal cases and carriers, insects and environment.
- **Endotoxins** are the lipopolysaccharide components of cell walls of Gram-negative bacteria and hence, by definition, Gram-positive bacteria do not have endotoxins.
- **Exotoxins** are produced by both Gram-positive and Gram-negative bacteria.
- **Biological effects of endotoxins** of bacteria include fever, hypotension, activation of complement cascade, disseminated intravascular coagulation and increased phagocytic activity of macrophages.
- Attenuated exotoxins of bacteria are called **toxoids**; they are not toxic but antigenic and hence used in **protective vaccines**.
- Pathogenic organisms can spread from one host to another by inhalation, ingestion, contact, contamination of wounds, blood-sucking arthropods, and by vertical transmission.
- Infectious diseases are responsible for 30% of the world's disease burden.
- Toxins of bacteria are classified as endotoxins and exotoxins.



Important Questions

1. What are the various modes of spread of infection? Describe each in brief giving suitable examples.
2. Distinguish between exotoxins and endotoxins in a tabulated form.
3. Define the terms 'pathogenicity' and 'virulence'. Name and discuss various determinants of virulence.
4. What is a carrier? Name and define various types of carriers.



Multiple Choice Questions

1. Exotoxins **do not** have which of the following characteristics?
 - (a) They are produced extracellularly.
 - (b) They are produced by both Gram-positive and Gram-negative bacteria.
 - (c) Cannot be converted into toxoids.
 - (d) They are heat-labile.
2. Which of the following bacteria is **not** invasive?
 - (a) *Streptococcus pyogenes*.
 - (b) *Salmonella* serotype Typhi.
 - (c) *Vibrio cholerae*.
 - (d) *Neisseria meningitidis*.
3. Which of the following infections is acquired from animals?
 - (a) Plague.
 - (b) Diphtheria.
 - (c) Meningococcal meningitis.
 - (d) Poliomyelitis.
4. Which of the following is **not** a congenital infection?
 - (a) Toxoplasmosis.
 - (b) AIDS.
 - (c) Candidiasis.
 - (d) Cytomegalovirus infection.
5. Japanese encephalitis is transmitted by:
 - (a) *Anopheles*.
 - (b) *Culex*.
 - (c) *Aedes*.
 - (d) *Mansonia*.
6. Which of the following bacteria **does not** produce enterotoxin?
 - (a) *Vibrio cholerae*.
 - (b) *Escherichia coli*.
 - (c) *Bacillus cereus*.
 - (d) *Bacillus anthracis*.
7. Which of the following infections is **not** spread by the respiratory route?
 - (a) Common cold.
 - (b) Measles.
 - (c) Mumps.
 - (d) Hepatitis B.
8. A carrier who acquires the organisms from another carrier is known as:
 - (a) a contact carrier.
 - (b) a paradoxical carrier.
 - (c) a convalescent carrier.
 - (d) a chronic carrier.
9. Which of the following fungi forms part of the normal flora, but may cause disease also?
 - (a) *Histoplasma*.
 - (b) *Blastomyces*.
 - (c) *Candida*.
 - (d) *Aspergillus*.
10. Which of the following pathogens is/are transmitted by rat flea?
 - (a) *Yersinia pestis*.
 - (b) *Rickettsia typhi*.
 - (c) *Hymenolepis diminuta*.
 - (d) All of the above.
11. Infectious agents can enter the body through which of the following routes?
 - (a) Inhalation.
 - (b) Ingestion.
 - (c) Inoculation.
 - (d) All of the above.



Answers

1. c 2. c 3. a 4. c 5. b 6. d 7. d 8. b 9. c 10. d 11. d

Immunity

Competency achievement: The student after reading the chapter should be able to:

- MI1.7:** Describe the immunological mechanisms in health.
- MI1.8:** Describe the mechanisms of immunity and response of the host immune system to infections.
- MI1.9(a):** Discuss the immunological basis of vaccines.
- BI10.5:** Describe antigens and concept involved in vaccine development.

Immunity refers to resistance of a host to pathogens and their toxic products. It is of two types:

- I. Innate immunity
 - (a) Non-specific
 - (b) Specific
 - II. Acquired immunity
 - (a) Active
 - Natural
 - Artificial
 - (b) Passive
 - Natural
 - Artificial
- } Species, racial, individual

I. INNATE IMMUNITY

It is due to genetic and constitutional make-up of an individual. Prior contact with microorganisms or their products is not essential. It may be specific against a particular organism or non-specific. Innate immunity may be further divided into species, racial or individual immunity.

Species immunity

It is total or relative resistance to a pathogen shown by all the members of a species. For example, all human beings are resistant to plant pathogens and many animal pathogens. Rat is strikingly resistant to diphtheria whilst guinea pig and man are highly susceptible. This is due to physical and biochemical differences between the tissues of different host species which determine if a pathogen can multiply in them.

Racial immunity

Within a species, there may be marked racial differences in resistance to infection, e.g. Algerian sheep is highly resistant to anthrax as compared to European sheep. In the USA, Negroes are more susceptible to tuberculosis than Whites. Racial differences in immunity are known to be genetic in origin. A hereditary (genetic) abnormality of red blood cells (sickling) confers immunity to infection by *Plasmodium falciparum* because such RBCs cannot be parasitized by these parasites. This may provide survival advantage to such individuals in malaria-infested areas.

Individual immunity

Different individuals in a race differ in their resistance to microbial infections. The genetic basis of individual immunity is apparent from the observation that if one homozygous twin develops tuberculosis, there is a 75% chance that the other twin will develop overt tuberculosis. In contrast, for heterozygous twins, there is only 33% chance that the second twin will contract overt disease.

Factors influencing innate immunity

1. Age

In general, very young and very old are more susceptible to infectious diseases than persons in other age groups. This appears to be due to the immaturity of immune system in very young and gradual waning of immune response in very old. Foetus *in utero* is protected from maternal infection by placental barrier. But some pathogens such as HIV cross this barrier leading to foetal infection, while others like *Toxoplasma gondii*, rubella, herpes simplex virus, varicella-zoster virus and cytomegalovirus lead to congenital malformations.

Newborn animals are more susceptible to experimental infection than adult animals, e.g. coxsackievirus causes fatal infection in suckling mice but not in adult mice. On the other hand, in many diseases such as measles, mumps, poliomyelitis and chickenpox, the clinical illness is more severe in adults than in children. This may be due to more active immune response which leads to greater tissue damage.

2. Hormonal influences and sex

There is an increased susceptibility to infection in endocrine disorders such as diabetes mellitus, hypothyroidism and adrenal dysfunction. The reason for this increased susceptibility is not known but it may be related to hormone activity. For example, glucocorticoids are anti-inflammatory agents. They inhibit the ability of phagocytes to ingest foreign particles. Staphylococcal, streptococcal and certain fungal infections such as candidiasis, aspergillosis and mucormycosis occur more frequently in diabetics. Pregnant women are more susceptible to microbial infections due to increased steroid levels during pregnancy. In general, incidence and death rate from infectious diseases is greater in males than in females. However, in case of hepatitis A and whooping cough, morbidity and mortality is higher in females than in males.

3. Nutritional factors

Both antibody-mediated and cell-mediated immunity are lowered in malnutrition. Protein calorie malnutrition:

- lowers C3 and factor B of the complement system,
- decreases the interferon response, and
- inhibits neutrophil activity.

Similarly, deficiency of vitamin A, vitamin C, folic acid and zinc predisposes to certain infections. Experimental studies have also shown that inadequate diet may be correlated with increased susceptibility to a variety of bacterial infections associated with decreased phagocytic activity and leucopenia.

Mechanism of innate immunity

1. Mechanical barriers and surface secretions

The intact skin and the mucous membranes provide a high degree of protection against pathogens. If skin is damaged, as in case of injury or burns, infections may be a serious problem. Skin is a very effective barrier because of unusual structure of outermost epithelial layer, which is composed mainly of keratin which is indigestible by most microorganisms, and thus protects the living cells of the epidermis from microorganisms and their toxins.

Even more important than this are the **fatty acids secreted by the sebaceous glands, propionic acid produced by the normal flora of the skin and high salt concentration in drying sweat**. Secretions from sebaceous glands contain both saturated and unsaturated fatty acids that kill many bacteria and fungi. The protective ability of these secretions varies at different stages of life. For example, ringworm of scalp caused by *Microsporum* and *Trichophyton* is difficult to cure in children, but after puberty it disappears without treatment. This appears to be due to change in amount and kind of fatty acids secreted by the sebaceous glands. Normal bacterial flora of skin and mucous surfaces help to prevent colonisation by pathogens.

We have a **specialized epithelial lining in our respiratory and gastrointestinal tract to minimize infection**. Mucous membrane is composed of specialized epithelial cells which secrete a sticky substance called mucus.

This traps dust. In respiratory tract there are also ciliated cells, which move the dust-laden mucus up and out of respiratory tract (**mucociliary escalator**) enabling it to be swallowed or coughed out. When swallowed they are destroyed in the stomach's highly acidic environment and digestive juices. Should a pathogen survive in stomach, it usually cannot penetrate mucous membrane lining the entire gastrointestinal tract and if it escapes the respiratory mucociliary escalator, it would be met by phagocytes lining the alveoli.

The mouth is constantly bathed in **saliva** which has an inhibitory effect on many microorganisms. Nevertheless viruses, bacteria, fungi and protozoa have developed strategies to circumvent these defences. Influenza virions, for example, have haemagglutinins on their surfaces. With the help of these they attach to the receptors on epithelial cells. Influenza virions also possess neuraminidase spikes on their surface. Neuraminidase splits up neuraminic acid of mucus thus helping them to establish contact of haemagglutinins with the receptors on epithelial cells because mucus forms a barrier between epithelial cells and microparasites. Nicotine from cigarettes can paralyse the mucociliary escalator. This exposes cigarette smokers to bacterial infection leading to chronic bronchitis.

Gastrointestinal tract

Four factors protect gastrointestinal tract from infection:

1. **Physical barrier** produced by mucus-secreting epithelial cells.
2. **Secretory IgA antibodies** produced here.
3. **Highly acidic environment** of stomach may hydrolyse microbial invaders.
4. Already established **normal flora** such as *Escherichia coli* serves to inhibit invasion and repopulation by pathogenic microorganisms. However, normal flora is a double-edged weapon. If body resistance is lowered it may lead to opportunistic infections.

Urogenital tract

Kidneys produce sterile urine which travels down the ureter to the bladder and passes out through the urethral opening. Although urethra has normal flora, invading microorganisms usually do not gain access to the bladder. It is mainly due to frequent flushing of urethra by sterile urine. However, if organisms have mechanism to attach to epithelial cells lining the tract even frequent flushing might not evacuate them. Because of short urethra, bladder infection is more common in females than in males.

An interesting relation exists between lactobacilli and *Candida* in vagina. Both normally colonize vagina and keep each other's growth under check. Both are able to use glycogen present in vagina. Lactobacilli produce lactic acid which lowers the pH, thereby inhibiting the growth of *Candida*. On the other hand, *Candida* inhibits growth of lactobacilli by limiting the food supply. Administration of broad-spectrum antibiotics kills lactobacilli releasing *Candida* from growth restraints.

Conjunctiva

Conjunctiva is continually being assaulted by microbe-laden dust. Whenever the dust hits the conjunctiva we blink and tears are produced. Tears mechanically wash away the particles and a hydrolytic enzyme, lysozyme, destroys most viruses and bacteria.

2. Humoral defence mechanisms

Many microbicidal substances are present in the tissues and body fluids. These are non-specific. There is no specific recognition of the microorganism and the response is not enhanced by re-exposure to the same microorganism. They are responsible for innate immunity. Following are the bactericidal substances present in the tissues and body fluids.

Lysozyme

This is a basic protein of low molecular weight (approximately 20,000 daltons) found in high concentrations in polymorphonuclear leucocytes as well as in most tissue fluids except CSF, sweat and urine.

Basic polypeptides

Several basic proteins, derived from tissues and blood cells, possess antibacterial activity. These include properdin, complement, beta-lysine, basic polypeptides such as leukins extracted from leucocytes and plakins from platelets, and interferon which possess antiviral activity. Complement plays an important role in the destruction of pathogenic microorganisms that invade the blood and tissues (*see* Chapter 12).

3. Cellular defence mechanisms

Microparasites that penetrate the physical barriers are confronted, in addition to humoral defence mechanism, by non-specific cellular defences. Cellular defence against microparasites is provided by **phagocytes** and a subpopulation of lymphocytes known as **natural killer (NK) cells**. Phagocytes are classified into microphages and macrophages. **Microphages** are polymorphonuclear leucocytes and **macrophages** consist of histiocytes which are the wandering amoeboid cells seen in tissues, fixed reticuloendothelial cells and monocytes of blood.

Phagocytic cells reach the site of inflammation in large numbers. They engulf, kill and digest bacteria. On the other hand, viral invasion is countered by NK cells. Residing in the peripheral lymphoid organs, NK cells recognize virus-infected cells, bind to them and subsequently lyse them. NK cells have also been implicated in host defence against cancers. They are thought to recognize the changes in the cell membranes of transformed cells in a mechanism similar to that used to combat virus infection. Fungi are confronted by polymorphonuclear leucocytes, macrophages and NK cells.

Phagocytic cell engulfs microparasite by extending pseudopodia around it. These fuse and microorganism is internalized into a vacuole (**phagosome**) which fuses with lysosomes found in the cell to form **phagolysosome** (Fig. 9.1). Microparasites are subjected to the lytic enzymes

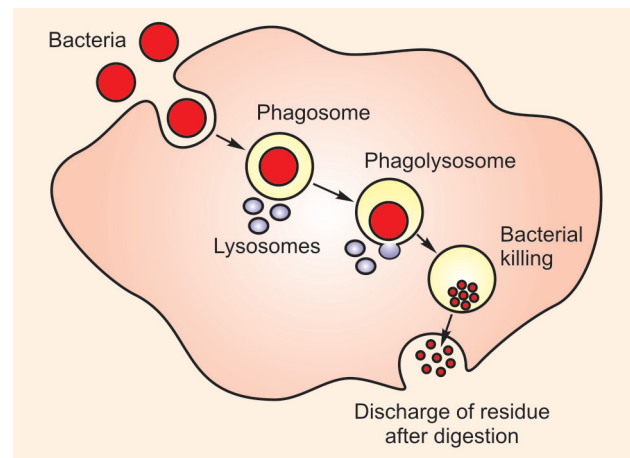


Fig. 9.1: Events of phagocytosis.

in the phagolysosome and are destroyed. The macrophages present in the walls of capillaries and vascular tissues in spleen, liver, lungs and bone marrow serve a very important role in clearing the blood stream of foreign particulate material such as bacteria. Some microorganisms such as **mycobacteria and brucellae resist intracellular digestion and may actively multiply inside the phagocyte**. Phagocytosis in such instances may help to disseminate infection to different parts of the body. However, these organisms can be digested by activated macrophages.

Eosinophils are polymorphonuclear leucocytes with cytoplasmic granules and bilobed nucleus. Their number in the blood of normal individuals is 3–5%. But **in patients with parasitic infections and allergies their number increases**. They are not efficient phagocytic cells. However, their granules possess molecules that are toxic to parasites. Large parasites such as helminths cannot be internalized. Therefore, they must be killed extracellularly. Eosinophils possess Fc receptors (that bind to the Fc fragment of different classes and subclasses of immunoglobulins) and complement receptors that bind the antibody and complement coated parasites. The granule contents of the eosinophils are then released into the space between the cell and the parasite. Eosinophil granules contain enzymes and toxic molecules that are active against helminths.

4. Fever

A rise in temperature following infection is a natural defence mechanism. It inhibits or kills the infecting organisms. Fever also stimulates production of interferon and helps in recovery from virus infection.

5. Inflammation

It is the cellular and vascular response to injury such as invasion by an infectious agent, exposure to a noxious chemical or physical trauma. The signs of inflammation are redness, swelling, heat, pain and disturbed or altered functions. Inflammation leads to vasodilation, increased vascular permeability and cellular infiltration. **Polymorphonuclear leucocytes** escape into the tissues by diapedesis and

accumulate in large numbers attracted by the chemotactic substances released at the site of injury. They then phagocytose microorganisms and their products. Because of increased vascular permeability, there is an **outpouring of plasma** which helps to dilute the toxic products present. In addition, plasma contains a number of non-specific (complement, properdin, beta lysin, leukins and plakins) and specific (antibodies) inhibitors.

II. ACQUIRED IMMUNITY

The resistance that an individual acquires during his life-time is known as acquired immunity. It is antigen-specific and may be antibody-mediated or cell-mediated. It is of two types—active immunity and passive immunity (Table 9.1 and Flowchart 9.1).

Both active and passive immunity may be further divided into natural and artificial.

Active immunity

This involves the active involvement of the person's own immune apparatus leading to the synthesis of antibodies and/or the production of immunocompetent cells (ICCs). It appears only after a **lag (latent) period**, i.e. the time required for generation of antibodies and ICCs. During development of active immunity there is often a negative phase during which the level of measurable immunity may actually be lower than before antigenic stimulus. This is due to antigen combining with pre-existing antibodies and lowering its level.

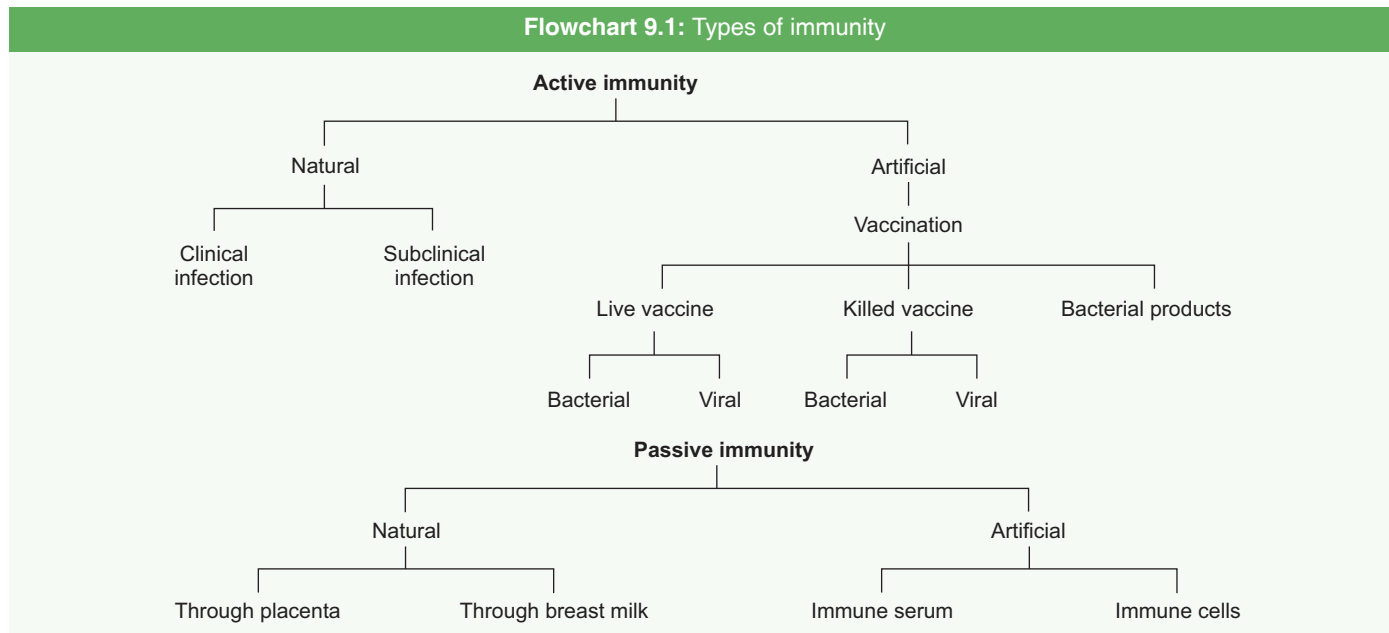
If an individual who has been actively immunized against an antigen, experiences the same antigen subsequently, the immune response occurs more quickly and more abundantly than during the first encounter. This is known as **secondary response**.

Immune system is able to retain the memory of a prior antigenic exposure for long periods and produces a secondary

Table 9.1: Differences between active and passive immunity

Active immunity	Passive immunity
- Produced actively by host's immune system as a result of antigenic stimulation. - Induced by infection or by contact with antigens. - Long lasting. - Immunity effective only after a lag period, i.e. time required for generation of antibodies and immunocompetent cells. - During development of active immunity there is often a negative phase during which the level of measurable immunity may actually be lower than before antigenic stimulus. This is due to antigen combining with the pre-existing antibodies and lowering its level. - Immunological memory present, therefore, subsequent challenge (secondary response) is more effective. - More effective and confers better protection. - Not applicable in immunodeficient individuals.	- Received passively by the host. No participation of host's immune system. - Conferred by administration of antibodies. - Transient. - Immunity effective immediately. - No negative phase. - No immunological memory. Subsequent administration of antibodies is less effective due to immune elimination. - Less effective and provides inferior immunity. - Applicable in immunodeficient individuals.

Flowchart 9.1: Types of immunity



type response when encountered with the same antigen. This is known as **immunological memory**.

Natural active immunity

Natural active immunity results either from a subclinical or clinical infection. A large majority of adults in the developing countries possess natural active immunity to poliomyelitis due to repeated subclinical infections with poliovirus during childhood. Some infections like diphtheria, whooping cough, measles and mumps induce long-lasting immunity. Others such as common cold and influenza confer immunity which lasts for a short time.

Artificial active immunity

This is the resistance induced by vaccines which are preparations of live or killed microorganisms or their products.

I. Bacterial vaccines

- (a) Live
 - BCG for tuberculosis
 - Ty21a for typhoid
- (b) Killed
 - TAB for enteric fever
 - Cholera
 - Pertussis
- (c) Bacterial products
 - Tetanus toxoid
 - Diphtheria toxoid
 - Capsular polysaccharide of meningococci
 - Capsular polysaccharide of *Haemophilus influenzae* type b

II. Viral vaccines

- (a) Live
 - Sabin vaccine for poliomyelitis or oral polio vaccine (OPV)
 - 17D vaccine for yellow fever
 - MMR vaccine for measles, mumps, rubella
 - Varicella-zoster
- (b) Killed
 - Salk vaccine for poliomyelitis
 - Neural and non-neural vaccines for rabies
 - Influenza
 - Hepatitis A
 - Hepatitis B
 - Japanese encephalitis

Live vaccines initiate a sort of mini infection without causing disease. The immunity following vaccination, therefore, parallels that following natural infection. However, it is of lower order than that induced by infection. Since live vaccines undergo limited multiplication in the body, therefore, number of organisms required in a dose is less; single doses may be sufficient and they are relatively cheaper. Live vaccines may be administered orally (e.g. Sabin vaccine for poliomyelitis). They provide more effective and more lasting immunity than killed vaccines. Some of them can be

given as combined vaccines, e.g. measles, mumps, rubella (MMR) vaccine.

Killed vaccines have the advantage of stability and safety. These can be given in combination in polyvalent vaccines, e.g. diphtheria, pertussis, tetanus (DPT) vaccine. These are, generally, less immunogenic than live vaccines and protection lasts only for a short period. Therefore, they have to be administered repeatedly. At least two doses are required. First dose is known as **primary** and subsequent doses as **booster doses**. In killed vaccines since the organisms are killed, therefore, larger number of these are required in each dose. Oral route for killed vaccines is, generally, not effective. Antibody response to killed vaccines is improved by addition of adjuvants, for example, aluminium phosphate adjuvant vaccine for cholera.

Passive immunity

The immunity that is transferred to a recipient in a ready-made form is known as passive immunity. Here the recipient's immune system plays no active role. There is no lag or latent period, the immunity is effective immediately after passive immunization. There is no negative phase. It confers only transient immunity lasting usually for days or weeks till the antibodies are metabolized and eliminated. There is no secondary type response. Rather subsequent administration of antibodies is less effective due to **immune elimination**.

Following first injection of antibody, its elimination is only by metabolic breakdown but during subsequent injections its elimination is much quicker because metabolic breakdown is combined with immune elimination as it combines with antibodies to horse serum that would have been produced following first injection. This happens when horse (foreign) serum is used. Immune elimination is not a problem when human serum is used. Because of its immediate action it is employed where instant immunity is required as in case of protection against tetanus, gas gangrene and diphtheria following exposure.

Natural passive immunity

This is the resistance transferred from mother to foetus through placenta. IgG antibodies can cross placental barrier to reach the foetus. After birth, immunoglobulins are passed to the newborn through the breast milk. Human colostrum is rich in IgA antibodies which are resistant to digestion in stomach and small intestine, hence confers immunity in the neonate up to 3 months of age.

Artificial passive immunity

This is the immunity transferred passively to the recipient by administration of antibodies. This is done by administration of hyperimmune sera of man or animals. For example, tetanus antitoxin is prepared in horses by active immunization of horses with tetanus toxoid, bleeding them and separating the serum. Similarly, diphtheria antitoxin and gas gangrene antitoxin are also prepared. However, since these antitoxins are foreign proteins and are liable to cause serious or even fatal hypersensitivity reactions, these should

be administered only after testing for hypersensitivity. After first administration, it is removed by metabolism and following subsequent injections by metabolism and immune elimination. Therefore, immunity conferred is short-lived.

Sera collected from patients convalescing from infectious diseases contain high levels of specific antibodies. Convalescent sera have, therefore, been employed for passive immunization against viral infections such as measles and rubella. Sera of healthy adults contain antibodies against infectious agents prevalent in a community. Therefore, sera from a large number of individuals can be collected and used for passive immunization. Placenta provides a convenient source of human immunoglobulins. Human immune serum does not lead to any hypersensitivity reaction, there is no immune elimination and its half-life is more than that of animal sera. However, with human serum there is a grave risk of transmission of human immunodeficiency virus and hepatitis B, C and D viruses.

Indications of passive immunization

- To provide immediate protection to a non-immune individual exposed to an infection, when there is insufficient time for active immunization, e.g. administration of tetanus antitoxin and gas gangrene antitoxin to a non-immune individual with crushing road-side injury, and administration of diphtheria antitoxin to a non-immune child exposed to diphtheria.
- Administration of anti-Rh(D) IgG to Rh-negative mother, bearing Rh-positive baby at the time of delivery to prevent Rh isoimmunization.
- For suppression of active immunity, when it is injurious, for example, administration of antilymphocytic serum for suppression of lymphocytes in transplantation surgery to suppress the immune response towards the transplant.

Combination of active and passive immunization may also be employed. For example, a person exposed to tetanus may be injected tetanus antitoxin on one arm and tetanus

toxoid on the other with separate syringes followed by full course of tetanus toxoid. Diphtheria antitoxin and diphtheria toxoid can also be practised similarly.

ADOPTIVE IMMUNITY

Injection of immunologically competent lymphocytes is known as adoptive immunity. Instead of whole lymphocytes, an extract of immunologically competent lymphocytes known as transfer factor can be used. This has been attempted in the treatment of lepromatous leprosy.

LOCAL IMMUNITY

This means immunity at a particular site, generally the site of invasion and multiplication of pathogen. For example, in case of poliomyelitis, parenteral vaccine provides systemic immunity. The antibodies neutralize virus only after blood invasion. It does not prevent multiplication of the virus at the site of entry, the gut mucosa, and its faecal excretion. However, when live oral vaccine is given it leads to local immunity. Similarly, live influenza vaccine administered intranasally provides local immunity while killed influenza vaccine evokes humoral antibody response. Local immunity is conferred by secretory IgA antibodies, produced locally by plasma cells, present on mucosal surfaces or in secretory glands.

HERD IMMUNITY

Overall level of immunity in a community is known as herd immunity. When a large number of individuals in a community (herd) are immune to a pathogen, the herd immunity to a pathogen is said to be satisfactory. When herd immunity is low, epidemics are likely to occur on the introduction of the pathogen. This is due to the fact that a larger number of individuals are susceptible.

KEY POINTS

- The **innate system** of immune defence consists of a formidable **barrier to entry**, and **second-line phagocytes and circulatory soluble factors**.
- **The cells which mediate immunity include lymphocytes and phagocytes**. Lymphocytes recognize antigens on pathogens. Phagocytes internalize pathogens and degrade them.
- The main phagocytic cells are **polymorphonuclear neutrophils** and **macrophages**.
- **Specificity and memory** are two essential features of acquired immunity. The immune system mounts a more effective immune response on second and subsequent encounters with a particular antigen.
- **Phagocytic cells** reach the site of inflammation in large numbers. They engulf, kill and digest bacteria. On the other hand, viral invasion is countered by NK cells.
- The **influx of polymorphs** and **increase in vascular permeability** constitute the potent antimicrobial acute inflammatory response.
- Natural infection may produce **lifelong protection** against reinfection with the same pathogen, with induction of memory T and B lymphocytes.
- Some infectious diseases can be prevented by vaccination in childhood with live attenuated or inactivated pathogens or their products.
- Injection of immunologically competent lymphocytes is known as **adoptive immunity**.
- Immunity at the site of invasion and multiplication of a pathogen is known as **local immunity**.
- Overall level of immunity in a community is known as **herd immunity**.

- Immune system exists to protect the body against threats from outside (pathogens) and inside (cancer).
- Components of innate immune system include *phagocytes*, *natural killer cells*, the *alternative complement pathway* and *inflammation*.



Important Questions

1. Discuss mechanisms of innate immunity.
2. What is the role of mechanical barriers and surface secretions in providing immunity against infections?
3. Differentiate between active and passive immunity in a tabulated form.
4. What is artificial active immunity? Describe it giving suitable examples.
5. Compare live and killed vaccines in a tabulated form.
6. Discuss briefly the vaccines.



Multiple Choice Questions

1. Passive immunization is indicated in:
 - (a) tuberculosis.
 - (b) enteric fever.
 - (c) diphtheria.
 - (d) rubella.
2. Which of the following vaccines is live?
 - (a) Salk.
 - (b) Sabin.
 - (c) TAB.
 - (d) Human diploid cell rabies vaccine.
3. Which class of immunoglobulins can cross the placenta?
 - (a) IgG.
 - (b) IgM.
 - (c) IgD.
 - (d) IgA.
4. Lysozyme is present in:
 - (a) tears.
 - (b) CSF.
 - (c) sweat.
 - (d) urine.
5. Which of the following cells are implicated in immunity against cancers?
 - (a) Natural killer cells.
 - (b) Neutrophils.
 - (c) Eosinophils.
 - (d) Platelets.
6. Concomitant immunity is seen in:
 - (a) tuberculosis.
 - (b) whooping cough.
 - (c) enteric fever.
 - (d) malaria.
7. Inflammation is influenced by histamine, which is released by:
 - (a) basophils.
 - (b) eosinophils.
 - (c) erythrocytes.
 - (d) platelets.
8. Which of the following microorganisms resist intracellular digestion and may actively multiply inside the phagocyte?
 - (a) *Corynebacterium diphtheriae*.
 - (b) *Mycobacterium tuberculosis*.
 - (c) *Clostridium tetani*.
 - (d) *Staphylococcus aureus*.
9. The cells responsible for killing intracellular viruses are:
 - (a) natural killer cells
 - (b) eosinophils.
 - (c) basophils.
 - (d) platelets.
10. Which of the following leads to longest-lasting immunity to an infectious agent?
 - (a) Naturally acquired passive immunity.
 - (b) Artificially acquired passive immunity.
 - (c) Naturally acquired active immunity.
 - (d) None of the above.



Answers

1. c 2. b 3. a 4. a 5. a 6. d 7. a 8. b 9. a 10. c

Antigens

Competency achievement: The student after reading the chapter should be able to:

BI10.5: Describe antigens and concept involved in vaccine development.

Antigens (*antibody generators*) are substances that can stimulate an immune response and, given the opportunity, react specifically by binding with the effector molecules (antibodies) and effector cells (lymphocytes) produced. Most antigens are proteins, but some are carbohydrates, lipids or nucleic acids. Some antigens are more immunogenic or capable of eliciting an immune response, than others. Some antigens such as proteins may possess a number of small chemical groups that are called antigenic determinants or **epitopes** which can bind specifically to antigen binding site (**paratope**) of the antibody molecule (Fig. 10.1) and T cell receptors. Each determinant can stimulate the formation of a particular kind of antibody or effector cell. Thus, a pure protein antigen may give rise to many distinct antibodies and effector cells. The size of epitope is around 25–35 Å and a molecular weight of 400–1000.

Incomplete antigen or hapten

This is a chemical substance of low molecular weight that cannot induce an immune response by itself. Nevertheless, haptens can induce a response if combined with larger molecules (normally proteins) which serve as carriers. In

response to hapten carried on carrier particle, antibodies are produced not only against the hapten but also against the carrier particle. Haptens are of two types.

1. Complex haptens

These can combine with specific antibodies to form precipitate, e.g. capsular polysaccharide of pneumococci.

2. Simple haptens

These combine with specific antibodies but no precipitate is formed (non-precipitating). This is due to univalent character of simple hapten, whereas complex haptens are polyvalent, since it is assumed that precipitation requires the antigens to have two or more antibody combining sites.

Immunogenicity

This refers to the ability of an antigen to stimulate an immune response.

Determinants of antigenicity

1. Size

Antigenicity depends upon the molecular weight. Generally, molecules with a molecular weight less than 5,000 are non-antigenic or feebly so. Low molecular weight substances may be rendered antigenic by absorbing them on large inert particles such as bentonite or kaolin.

2. Foreignness

Only antigens which are foreign to the individual (non-self) induce an immune response because host distinguishes self from non-self and normally does not respond to self. The healthy body is immunologically tolerant to nearly all self antigens, that would be immunogenic in a foreign host. However, under certain circumstances tolerance may be broken leading to **autoimmunity**. *Antigenicity of a substance is related to the degree of its foreignness.* Antigens from other individuals of the same species are less antigenic than those from other species.

3. Chemical nature

Proteins and polysaccharides are most antigenic. Lipids and nucleic acids are less antigenic. Their antigenicity is enhanced

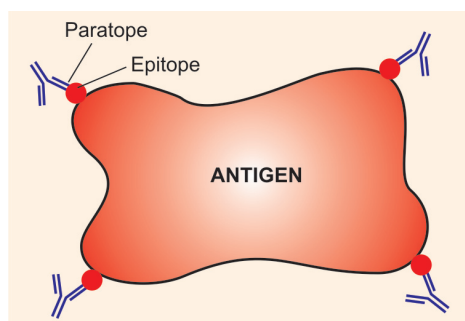


Fig. 10.1: Epitopes of antigen and paratopes of antibody.

by combination with proteins. However, all proteins are not antigenic. A well-known exception is gelatin. The presence of an aromatic radical appears to be essential for antigenicity and the absence of aromatic amino acids such as tyrosine in gelatin is responsible for its non-antigenicity.

4. Susceptibility to tissue enzymes

Only those substances which can be metabolized and are susceptible to the action of tissue enzymes behave as antigens. Antigens introduced into the body are degraded by the host into the fragments of appropriate size containing antigenic determinants. Phagocytosis and intracellular enzymes appear to play an essential role in breaking down antigens into immunogenic fragments. Substances insusceptible to tissue enzymes such as polystyrene latex and synthetic polypeptides which are not metabolized in the body, are not antigenic.

Antigenic specificity

It is determined by chemical grouping and acid radicals. Antigenic specificity varies with the position of antigenic determinant, i.e. whether it is in *ortho*, *meta* or *para* positions. However, antigenic specificity is not absolute. Cross reactions can occur between antigens which bear stereochemical similarities.

Species specificity

Tissues of all individuals in a species contain species-specific antigens. However, some degree of cross reactivity is seen between antigens from related species. **Species-specific antigens possess forensic applications in the identification of species of blood and seminal stains.**

Isospecificity

Isoantigens are antigens found in some but not all members of a species. On the basis of isoantigens a species may be divided into different groups. The best example of isoantigens is human blood group antigens on the basis of which all humans can be divided into different groups—A, B, AB and O. Each of these groups may be further divided into Rh-positive or Rh-negative. **This carries clinical importance in blood transfusion, isoimmunization during pregnancy and disputed paternity.**

Histocompatibility antigens

These are the antigens present on the cells of each individual of a species. Histocompatibility typing is essential in organ/tissue transplantation from one individual to another within a species. These antigens are associated with plasma membrane of tissue cells and are responsible for evoking immunological response against graft unless it is antigenically identical to that of the recipient. These antigens are encoded by genes known as *histocompatibility* genes which collectively constitute **major histocompatibility complex (MHC)**. These are located on short arm of chromosome 6. MHC products present on the surface of leucocytes are known as **human leucocyte-associated (HLA) antigens**. These have been studied extensively in organ transplantation. Major histocompatibility antigens in man and mouse are known as HLA and H2, respectively (see Chapter 19).

Autospecificity

Autologous or self antigens are ordinarily non-antigenic. However, hidden or sequestered antigens that are not normally found free in circulation or tissue fluids are not recognized as self antigens. For example, **lens protein** which is normally confined within the capsule of the lens, and antigens that are absent during the embryonic life and develop later, such as **spermatozoa**, are also not recognized as self antigens. But if these antigens are released into the tissues, as for instance following injury to lens or damage to the testis, antibodies are produced against them. This is one of the mechanisms of pathogenesis of autoimmune diseases. Cells or tissues may undergo **antigenic alteration** as a result of infection or irradiation and may thus become immunogenic leading to **autoimmunity**.

Organ specificity

Some organs such as brain, kidney and lens protein of different species share the same antigens. These are known as organ-specific antigens. The **neuromuscular complications** following antirabic vaccination, with neural vaccines, are a consequence of brain-specific antigens shared by sheep and man.

Heterogenetic (heterophile) specificity

Same or closely related antigens occurring in different biological species, classes and kingdoms are known as heterogenetic or heterophile antigens. The best example of such heterophile antigens is the **Forssman antigen** which is a lipid carbohydrate complex widely distributed in man, animals, birds, plants and bacteria. It is absent in rabbits, therefore, anti-Forssman antibody can be prepared in these animals. Examples of tests based on the principle of heterophile antigens used in diagnostic serology are as under.

(i) Weil-Felix reaction

It is an agglutination test in which patient sera are tested for agglutinins to O antigens of non-motile strains of *Proteus* OX2, OX19 and OXK. Cross reaction between O antigen of these strains of *Proteus* and certain rickettsial antigens is the basis of this test.

(ii) Paul-Bunnell test

In patients with infectious mononucleosis heterophile antibodies appear in the serum of the patient. These antibodies agglutinate sheep erythrocytes. This test is known as Paul-Bunnell test.

(iii) Cold agglutinin test

Agglutination of human O group erythrocytes at 4°C by the sera of patients suffering from primary atypical pneumonia.

(iv) Agglutination of *Streptococcus MG*

Agglutination of *Streptococcus MG* by the sera of the patients of primary atypical pneumonia.

Superantigens

Superantigens is a class of molecules that can interact with antigen-presenting cells (APCs) and T cells in non-specific manner. This activity does not involve the endocytic processing required for typical antigen presentation but instead occurs by concurrent association with MHC class II molecules of the APCs and the V β domain of the T cell

receptor (Fig. 10.2). This interaction activates a large number of T cells (10%) than conventional antigens (about 1%), explaining the massive cytokine expression and immunomodulation. Various superantigens include staphylococcal enterotoxins, staphylococcal toxic shock syndrome toxin, staphylococcal exfoliative toxin, streptococcal pyrogenic exotoxin and some viral proteins.

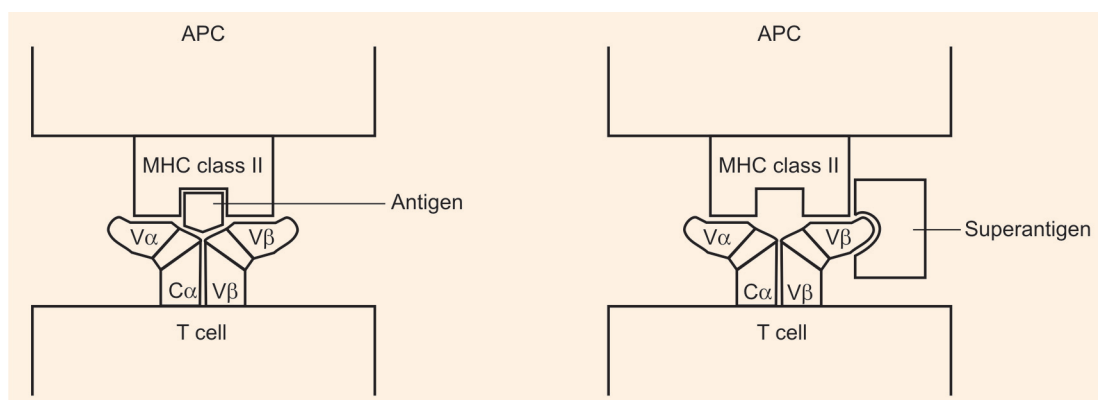


Fig. 10.2: Binding of antigen and superantigen by MHC and T cell receptor.

KEY POINTS

- **Antigens** are substances that can stimulate an immune response and, given the opportunity, react specifically by binding with the effector molecules (antibodies) and effector cells (lymphocytes).
- Chemical groups which can bind specifically to antigen binding site (*paratope*) of the antibody molecule are called **epitopes** (antigenic determinants).
- A substance capable of eliciting immune response is known as **immunogen**.
- Same or closely related antigens occurring in different biological species, classes and kingdoms are known as **heterogenetic** or **heterophile antigens**.
- **Superantigen** is a class of molecules that can interact with **antigen-presenting cells** and **T cells** in non-specific manner.



Important Questions

1. What is an antigen? Discuss briefly various determinants of antigenicity.
2. What are heterophile antigens? Name various serological tests which are based on the principle of heterophile antigens. Discuss briefly their principles and uses.
3. What are haptens? Distinguish between the terms epitope and paratope.



Multiple Choice Questions

1. The example of a serological test based on heterophile antigens is:
 - (a) Paul-Bunnell test.
 - (b) Widal test.
 - (c) VDRL test.
 - (d) antistreptolysin O test.
2. The determinants of antigenicity is/are:
 - (a) size of the antigen.
 - (b) foreignness.
 - (c) chemical nature.
 - (d) all of the above.
3. An antigen that occurs in various tissues of different species is referred to as:
 - (a) heterophile antigen.
 - (b) isoantigen.
 - (c) autoantigen.
 - (d) hapten.



Answers

1. a
2. d
3. a

Antibodies

Competency achievement: The student after reading the chapter should be able to:

MI1.10(a): Describe the immunological mechanisms of hypersensitivity and discuss the laboratory methods used in detection of hypersensitivity.

MI1.10(b): Describe the immunological mechanisms of autoimmunity.

MI1.10(c): Describe immunological mechanisms in immunological disorders in immunodeficiency states.

Antibodies or immunoglobulins (Igs) are γ globulins which are produced in response to antigenic stimulation. These react specifically with the antigens which stimulated their production. Igs are produced by plasma cells and to some extent by lymphocytes also. All antibodies are Igs, but all Igs (e.g. myeloma proteins) are not antibodies. On the basis of physicochemical and antigenic structure Igs can be divided into five distinct classes or isotypes namely IgG, IgA, IgM, IgD and IgE. They differ from each other in size, charge, carbohydrate content and amino acid composition. Within certain classes there are subclasses that show slight differences in structure and function from other members of the class. If injected into animals they induce the formation of antibodies that can be used to differentiate between the different isotypes serologically.

ANTIBODY STRUCTURE

IgG has been studied extensively and serves as a model of basic structural unit of all Igs. It is a Y-shaped four polypeptide chain molecule. Of the four chains, two each are light (L) and heavy (H). These are held together by disulphide bonds (Fig. 11.1). L chain has a molecular weight of 25,000 daltons and H chain 50,000 daltons. H chains are structurally and antigenically distinct for each class and are designated with Greek letters α (alpha), δ (delta), ϵ (epsilon), γ (gamma) and μ (mu) in IgA, IgD, IgE, IgG and IgM, respectively. L chains are of two types— κ (kappa) and λ (lambda). A molecule of Ig may have either κ or λ chains

but never both together. κ and λ chains occur in a ratio of about 2:1 in human serum.

IgG when treated with proteolytic enzyme papain in the presence of cysteine cleaves it into three fragments. Two identical fragments (45,000 daltons each) still possess the antigen-binding sites and are thus named **fragment antigen binding (Fab)**. These two fragments represent bivalency of IgG molecule. The third fragment (50,000 daltons) which lacks the ability to bind to antigen can be crystallized. It is, therefore, known as **fragment crystallizable (Fc)**.

Functions of Fc

- Binds complement leading to complement fixation.
- Binds to cell receptors (FcRs).
- Determines passage of IgG across the placental barrier.
- Determines skin fixation and catabolic rate.
- Antigenic determinants that distinguish one class of antibody from another are also located on Fc fragment.

Treatment of the IgG antibody molecule with proteolytic enzyme pepsin cleaves H chains on the carboxyterminal side of the interchain disulphide bonds of the hinge region. Therefore, 2 Fab fragments remain united (100,000 daltons). This fragment is designated as **F(ab')₂** with two antigen-binding sites. It is about 10% larger than the two Fab fragments from a papain digestion of antibody. Pepsin also degrades part of the Fc portion to small peptides and leaves a dimer of the carboxyterminal quarter of the chain, termed **pFc'**.

When IgG is treated with reducing agent such as mercaptoethanol in the presence of urea, the disulphide bonds are reduced releasing four peptide chains—two heavy and two light.

Immunoglobulin domains

Two H chains are always identical in a given molecule and the same is true of L chains. Each H chain of IgG contains 440 amino acids while each L chain contains 220 amino acids. H chain has four domains of 110 amino acids each, while L chain has two domains of 110 amino acids each (Fig. 11.2). The antigen combining sites of the molecule are at its

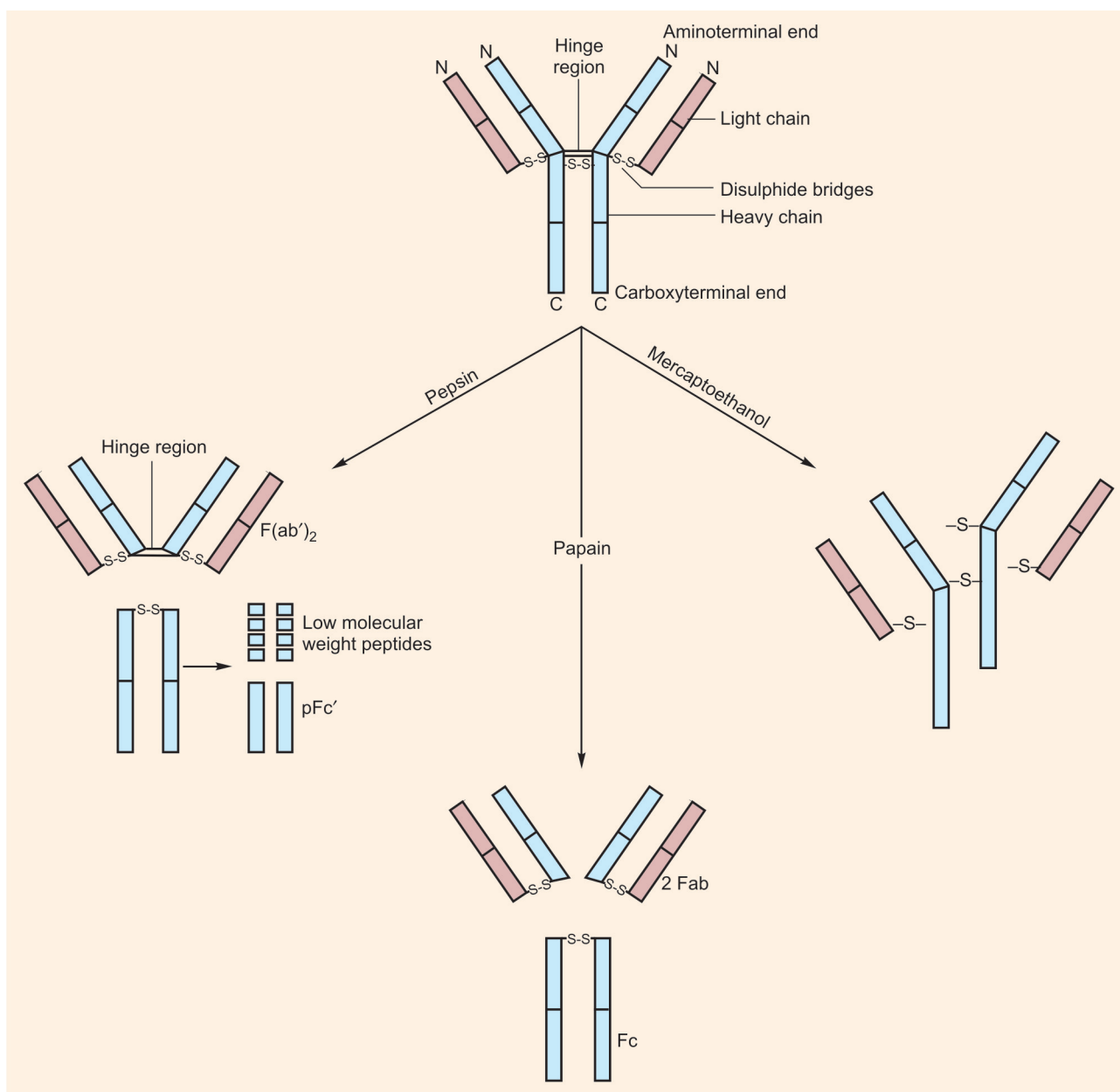


Fig. 11.1: Basic immunoglobulin structure.

aminoterminal end. These are composed of both H and L chains. Of the 220 amino acids, those that constitute carboxy-terminal half of L chain occur in a constant sequence. This part of the chain is called **constant region** (C_L). Only two sequence patterns are seen in constant region of κ and λ chains. On the other hand, amino acid sequence in aminoterminal half of the L chain is highly variable; the variability determines the immunological specificity of the antibody molecule. It is, therefore, called **variable region** (V_L).

A similar pattern is seen in H chains. The variable region of H chain, however, is only 25% as long as constant region. The variable region of H chain like that of L chain has highly variable sequence of amino acids and is known as V_H . The constant region of H chain is divided into three portions— C_{H1} , C_{H2} and C_{H3} . The infinite range of specificity of Igs depends upon the variability of amino acid sequences at the

variable regions of H and L chains which form antigen combining sites.

Fd piece

It is the portion of H chains present in Fab fragment. H chains carry a carbohydrate moiety which is distinct for each class of immunoglobulins.

Each Ig peptide chain has internal disulphide links in addition to interchain disulphide bonds which bridge H and L chains. These intrachain disulphide bonds form loops in the peptide chain and each of the loops is completely folded to form a globular domain and each domain has its separate function. Variable region domains V_L and V_H are responsible for the formation of a specific antigen-binding site. C_{H2} region in IgG binds C1q in the classical complement sequence and C_{H3} domain mediates adherence to monocyte

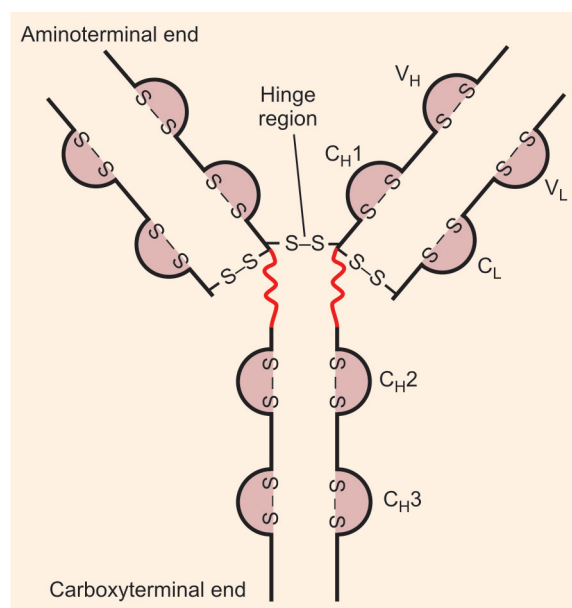


Fig. 11.2: Variable and constant domains of immunoglobulin molecule.

surface. The area of H chain in the C region between C_{H1} and C_{H2} is the **hinge region**. It is more flexible and is more exposed to enzymes and chemicals.

IMMUNOGLOBULINS AS ANTIGENS

Antibodies are glycoproteins in nature, therefore, they are good antigens. They can induce immune response even in a closely related host. The immune response is generated due to the antigenic determinants (epitopes) present on constant as well as variable regions of heavy and light chains. The antigenic determinants on immunoglobulin molecules fall into three major categories—isotypic, allotypic, and idiotypic determinants, which are located in characteristic portions of the molecule (Fig. 11.3).

1. Isotypic determinants

Isotypic determinants are located in the C domains of Ig chains in all individuals. Each isotype is encoded by a separate constant-region gene, and all members of a species carry the same collection of constant-region genes. Within a species, each normal individual will express all isotypes in the serum.

2. Allotypic determinants

These are distinct amino acid residues located primarily in γ and α H chains and κ L chains. They are possessed by some but not all individuals of a particular species. Allotypic determinants reflect genetic polymorphism of Igs within one species. Thus, individuals of a given species possess a given Ig class that either does or does not carry certain allotypic determinants. Antibodies can be formed against an allotypic determinant by injecting Igs into another member of the species that does not possess the antigen. In humans a number of allotypic markers have been discovered on Igs.

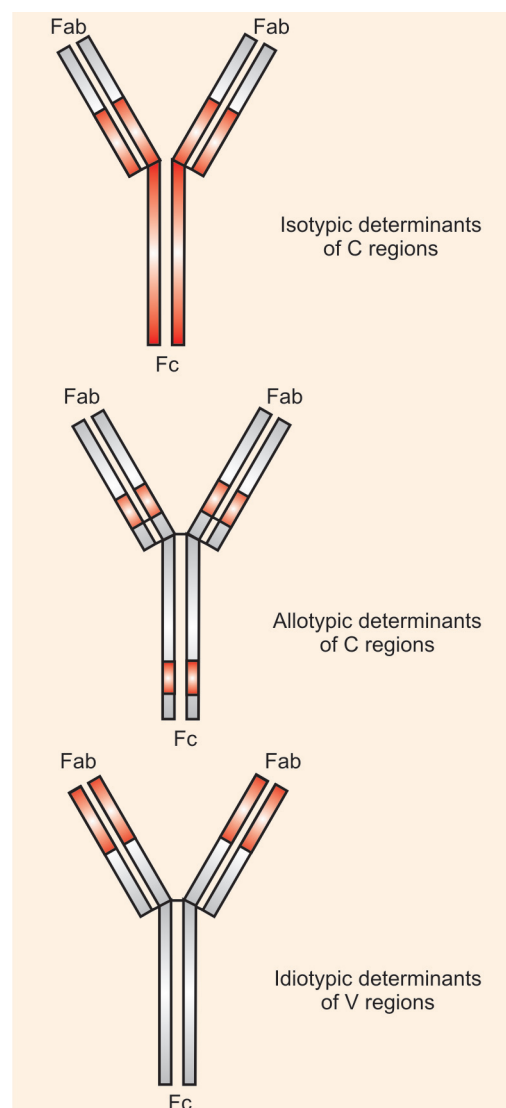


Fig. 11.3: Isotypic, allotypic and idiotypic determinants of immunoglobulins.

3. Idiotypic determinants

These are located on the V regions of L and H chains at or near the antigen-combining sites. These antigenic determinants are also called idiotopes analogous to epitope of classical antigens.

IMMUNOGLOBULIN CLASSES

Human serum contains five classes of immunoglobulins—IgG, IgA, IgM, IgD and IgE. Table 11.1 shows their differentiating features.

Immunoglobulin G

This is the most abundant class of Ig in the body constituting approximately 75% of the total Igs. This is distributed equally within the intravascular and extravascular pools. Very little IgG is produced during the early stages of the primary response to antigen, but it is the major form of antibody produced during the secondary response. It is not synthesized by the foetus in any significant amount. IgG is also most

Table 11.1: Properties of various immunoglobulin classes

	IgG	IgA	IgM	IgD	IgE
1. Molecular weight in kDa	150	160,385*	900–1,000	180	190
2. Sedimentation coefficient (s)	7	7, 11	19	7	8
3. Carbohydrate content (%)	3	8	12	13	12
4. Heavy chain	$\gamma_1, \gamma_2, \gamma_3, \gamma_4$	α_1, α_2	μ	δ	ϵ
5. Light chain	κ or λ	κ or λ	κ or λ	κ or λ	κ or λ
6. Serum concentration (mg/ml)	12	2	1.2	0.03	0.00004
7. Half-life (days)	21	6	5	3	2
8. Complement binding	Classical pathway	Alternative pathway	Classical pathway	None	None
9. Binding to tissue	Heterologous	None	None	None	Homologous
10. Secretion from serous membranes	No	Yes*	No	No	Yes
11. Placental passage	Yes	No	No	No	No
12. Heat stability (56°C)	Yes	Yes	Yes	Yes	No

* Secretory IgA.

commonly seen **myeloma protein**. It has a half-life of 21 days. The normal serum concentration of IgG is about 12 mg/ml. It is a glycoprotein with a molecular weight of 150,000 daltons.

There are **four subclasses** of human IgG (IgG1, IgG2, IgG3 and IgG4). Each subclass possesses a distinct type of γ chain which can be identified with specific antiserum. They constitute about 59%, 30%, 8% and 3%, respectively of the total human IgG. All normal humans possess all four subclasses of IgG, just as they possess all classes of Igs. IgG binds complement in classical pathway. IgG3 is most effective in binding complement followed by IgG1 and IgG2. It can bind to protein A (from *Staphylococcus aureus*) and protein G (from group G streptococci). *IgG is the only class of Igs that can cross the placenta and is responsible for the protection of the infant during first few months of life. However, subclass IgG2 does not cross the placenta.* IgG is also found, along with IgA, in milk during the first few weeks after birth, providing additional protection if the infant is breast-fed.

Macrophages and monocytes bear Fc receptors (FcRs) which bind to the Fc portion of IgG1 and IgG3 in C_H3 domain. Such binding permits these cells to exhibit antibody-dependent cellular toxicity. IgG usually exhibits high affinity for antigens leading to efficient neutralization of toxins. Among null cells, a distinct subpopulation of cytotoxic cells has been recognized which also possesses FcRs for Fc part of IgG. They are capable of lysing or killing target cells sensitized with IgG. They are known as **killer cells**. They are responsible for **antibody-dependent cell-mediated cytotoxicity (ADCC)**. Platelets also possess FcRs for Fc portion of IgG leading to aggregation, degranulation and release of histamine. IgG is the only Ig which has the property of fixing to guinea pig skin.

Catabolism of IgG is unique in that it varies with its serum concentration. When its level is raised, as in chronic malaria, kala-azar or myeloma, the IgG synthesized against

a particular antigen will be catabolised rapidly and may result in the particular antibody deficiency. Conversely, in hypogammaglobulinaemia, the IgG given for treatment will be catabolised slowly.

IgG participates in most immunological reactions such as complement fixation, precipitation and neutralization of toxins and viruses. Passively administered IgG suppresses the homologous antibody synthesis by a feedback process. This property is utilized for prevention of isoimmunization of Rh-negative mother bearing Rh-positive baby by administration of anti-Rh (D) IgG at the time of delivery.

Immunoglobulin M

It is so named because it is a macroglobulin at least five times larger than IgG. It is a glycoprotein with molecular weight of 900,000–1,000,000 daltons (millionaire molecule). It is present on the surface of virtually all uncommitted B cells. About 10% of normal serum Igs consist of this class. The normal serum level of IgM is 1.2 mg/ml. It has a half-life of about 5 days. IgM normally exists as a pentamer, consisting of 5 Ig subunits (Fig. 11.4).

In contrast to IgG, IgM remains almost exclusively in the serum and is not usually found extravascularly in body cavities or secretions. Therefore, IgM is believed to be responsible for protection against blood invasion by microorganisms. Pentameric IgM is apparently too large to cross the placenta. The H μ chain has four C_H domains rather than three as seen in H chains of IgG. H chains are held together by disulphide bonds. There is an additional peptide chain called the **joining (J) chain**. The J chain may be largely responsible for the polymerization process, which occurs shortly before the molecule is secreted by plasma cell.

IgM contains 10 Fab fragments, and thus 10 antigen-binding sites. Therefore, theoretically it can bind to 10 antigen molecules. However, it appears that many antigens are so

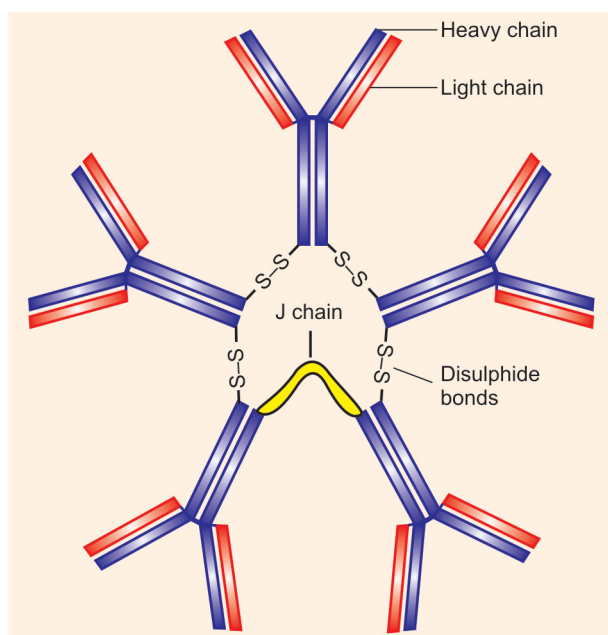


Fig. 11.4: IgM immunoglobulin.

large that when bound to one site, they physically prevent the binding of another antigen molecule to an adjacent binding site. Thus, generally, IgM is capable of binding as few as five molecules of antigen.

Phylogenetically IgM is the oldest Ig class. It is usually the first antibody to appear following stimulation by an antigen. However, IgM synthesis is usually not prolonged, and IgG antibodies soon become the most prevalent class. IgM is also the earliest to be synthesized by foetus beginning by about **20 weeks of gestation**. As it cannot cross the placental barrier, the presence of IgM in the foetus or newborn indicates intrauterine infection. Its detection is, therefore, useful for the diagnosis of congenital syphilis, rubella, HIV infection and toxoplasmosis. IgM antibodies are relatively short-lived, hence their demonstration in the serum indicates recent infection. Treatment of serum with 0.12 M 2-mercaptoethanol selectively destroys IgM without affecting IgG antibodies. This provides a simple method for differential estimation of IgG and IgM antibodies.

IgM is much more efficient than IgG in its ability to fix complement, promoting lysis and death of most Gram-negative bacteria. This greater efficacy is due to the fact that complement may bind to several Fc regions of pentameric IgM simultaneously thus initiating complement cascade and target cell lysis with a single molecule.

Isohaemagglutinins (anti-A and anti-B), and antibodies to *Salmonella* serotype Typhi O antigen and Wassermann reaction antibodies in syphilis are usually IgM.

Immunoglobulin A

The basic structure of IgA is similar to that of IgG. It contains two identical light chains (either κ or λ) and two heavy α chains. It is the second most abundant class, constituting about 15% of human serum Igs where it exists as a monomeric Ig. More important form is the dimeric form,

known as **secretory IgA (sIgA)**. It is the predominant class of Igs in secretions such as milk, tears, nasal secretions, saliva, perspiration, genitourinary secretions and seromucous secretions.

On mucus surfaces sIgA form an antibody paste and is believed to play an important role in local immunity against respiratory and intestinal pathogens. sIgA is relatively resistant to the digestive enzymes and reducing agents. Many infectious organisms cause disease by attaching to glycoproteins on the surface of epithelial cells of secretory gland. If this adhesion is sufficiently strong, the organism will divide, establish a colony and cause disease by any of a number of mechanisms, e.g. secretion of toxins that cause local and systemic injury. Secretory IgA when present in the secretions prevents attachment of organisms to epithelial cells thus preventing adhesion, colonization and infection.

Serum IgA is principally a monomeric 7S molecule with a molecular weight of 160,000 daltons. Secretory IgA is synthesized by plasma cells in the subepithelial tissue and secreted as a dimer containing four heavy chains, four light chains and one J chain which is similar to J chain found in pentameric IgM (Fig. 11.5). sIgA also possesses an additional structural unit called secretory component (SC). It is synthesized not in lymphoid cells but in epithelial cells of glands of intestine, and the respiratory tract and is attached to IgA molecules at their Fc portions producing 11S dimer with a molecular weight of 385,000 daltons. sIgA is relatively resistant to digestive enzymes, which may be due to the secretory component. IgA does not fix complement in classical pathway but can activate the alternative complement pathway. It promotes phagocytosis and intracellular killing of microorganisms.

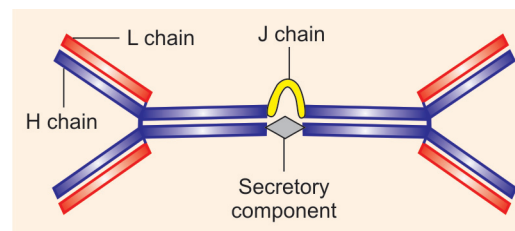


Fig. 11.5: Secretory IgA.

There are two subclasses of IgA in humans—IgA1 and IgA2. In serum, IgA1 constitutes 80–90% of IgA while sIgA consists of about equal amounts of the two subclasses.

Certain streptococci and pathogenic *Neisseria* produce proteases that specifically cleave the heavy chain of IgA1. IgA2 is resistant to such cleavage because it has a shorter hinge region and lacks the proline-rich site cleaved by the proteases.

Immunoglobulin D

Like other monomeric antibodies IgD is composed of two light and two heavy chains. The latter are designated as δ chains. It contains about 13% carbohydrate. Its molecular weight is 180,000 daltons. It does not bind complement. It

does not cross placenta and does not bind to cells via Fc region. IgD is present on the surface of B lymphocytes which are destined to differentiate into antibody-producing plasma cells and its serum concentration is very low (0.03 mg/ml). Reaction of antigen with surface immunoglobulin may lead to cell differentiation and antibody synthesis.

Immunoglobulin E

It resembles IgG structurally. Its molecular weight is 190,000 daltons. Its half-life is about 2 days. It does not fix complement or cross placental barrier. In contrast to other Igs it is heat-labile and gets inactivated by heating it at 56°C for 30 minutes. It is susceptible to 2-mercaptoethanol. It is chiefly produced in the linings of the respiratory and intestinal tracts. It is present in extremely low concentrations (0.00004 mg/ml) in the serum. But raised serum levels are seen in atopic (type I hypersensitivity) conditions like asthma and hay fever.

Most of a person's IgE is fixed to the surface of mast cells and basophils, and mediates type I hypersensitivity.

It has been observed that IgE levels may rise following infections with parasites especially helminths. It has been suggested that mast cell-bound IgE reacts with antigens on the parasite followed by release of histamine. This results in increased vascular permeability followed by influx of plasma and cells (particularly eosinophils) and destruction of parasite. IgE mediates Prausnitz-Kustner reaction.

From available information it appears that:

- IgG : Protects the body fluids
- IgA : Protects the body surfaces
- IgM : Protects the blood stream

IgE : Mediates type I hypersensitivity

IgD : Present on the surface of B lymphocytes which are destined to differentiate into antibody-producing plasma cells

ABNORMAL IMMUNOGLOBULINS

Abnormal immunoglobulins are structurally similar proteins. These are found in serum in certain pathological conditions such as **multiple myeloma** and sometimes even in healthy persons. **Bence-Jones (BJ) protein** was the earliest abnormal protein described in 1847. It is typically found in multiple myeloma. These proteins are light chains of immunoglobulins, hence occur as κ or λ forms. But in a patient, it may occur as κ or λ forms but never in both forms. It can be identified in urine by its characteristic property of coagulation when heated to 60°C but redissolving at 70°C.

Multiple myeloma may affect plasma cells synthesizing IgG, IgA, IgD and IgE. Similar involvement of IgM-producing cells is known as *Waldenstrom's macroglobulinemia*. In this condition, there is excessive production of the respective myeloma proteins (M proteins) and that of their light chains (BJ proteins).

Heavy chain disease is a different disorder. It is a lymphoid neoplasia characterized by the overproduction of the Fc parts of the heavy chains of immunoglobulins.

Cryoglobulinemia is the condition in which the serum from the patient precipitates on cooling and redissolves on warming. It may not always be associated with disease but is often found in patients with myelomas, macroglobulinemias, and autoimmune conditions such as systemic lupus erythematosus. Most cryoglobulins consist of either IgG or IgM or both.

KEY POINTS

- **Antibodies** or immunoglobulins are γ globulins which are produced in response to antigenic stimulation. These react specifically with the antigens which stimulated their production.
- **The immunoglobulins have a basic unit of two light chains and two heavy chains.**
- **There are five classes of antibody—IgG, IgA, IgM, IgD and IgE.**
- **Circulating antibodies** recognize antigen in serum and tissue fluids.
- **The infinite range of specificity of immunoglobulins** depends upon the **variability of amino acid sequences** at the variable regions of heavy and light chains which form **antigen combining sites**.
- Antibodies neutralize antigens, induce killing of target cells by complement and natural killer cells and opsonize particles for phagocytosis.
- IgG is the **most abundant** class of immunoglobulins in the body constituting approximately 75% of total immunoglobulins.
- **Joining (J) chain** is present in **IgM** and **secretory IgA**.
- **Secretory IgA** is relatively **resistant to digestive enzymes**, which may be due to the **secretory component** of this immunoglobulin.
- In contrast to other immunoglobulins, **IgE** is **heat-labile** and gets inactivated by heating at 56°C for 30 minutes.

Important Questions

1. What is an antibody? Draw labelled diagram of IgG, IgA and IgM.
2. Name various classes of immunoglobulins and describe structure and functions of IgG, IgA and IgM.
3. Tabulate differences between IgG, IgA and IgM.



Multiple Choice Questions

1. The function/s of Fc fragment of IgG is/are:
(a) it binds complement.
(b) it is related to passage of IgG across the placental barrier.
(c) it determines catabolic rate.
(d) all of the above.
2. Type I hypersensitivity reaction is mediated by:
(a) IgE. (b) IgG.
(c) IgM. (d) IgD.
3. Which immunoglobulin class is heat-labile?
(a) IgG.
(b) IgM.
(c) IgA.
(d) IgE.
4. Which class of immunoglobulin can bind to mast cells and basophils?
(a) IgA.
(b) IgM.
(c) IgD.
(d) IgE.
5. First antibody to appear following stimulation by an antigen is:
(a) IgM.
(b) IgG.
(c) IgA.
(d) IgE.
6. Most abundant class of immunoglobulin in body is:
(a) IgG.
(b) IgM.
(c) IgD.
(d) IgE.
7. J chain is present in:
(a) IgG.
(b) IgM.
(c) IgD.
(d) IgE.
8. The earliest immunoglobulin to be synthesized by the foetus is:
(a) IgA.
(b) IgD.
(c) IgG.
(d) IgM.
9. Isohaemagglutinins and antibodies to *Salmonella* serotype Typhi O antigen and Wassermann reaction antibodies in syphilis are usually:
(a) IgA.
(b) IgD.
(c) IgG.
(d) IgM.
10. IgM is the earliest class of immunoglobulin to be synthesized by foetus beginning by about:
(a) 15 weeks of gestation.
(b) 20 weeks of gestation.
(c) 25 weeks of gestation.
(d) 30 weeks of gestation.
11. Which subclass of immunoglobulin G (IgG) does not cross the placental barrier?
(a) IgG1.
(b) IgG2.
(c) IgG3.
(d) IgG4.



Answers

1. d 2. a 3. d 4. d 5. a 6. a 7. b 8. d 9. d 10. b 11. b

The Complement System

Competency achievement: The student after reading the chapter should be able to:

MI1.10(a): Describe the immunological mechanisms of hypersensitivity and discuss the laboratory methods used in detection of hypersensitivity.

MI1.10(c): Describe immunological mechanisms in immunological disorders in immunodeficiency states.

MI2.2: Describe etiopathogenesis, clinical features and discuss diagnostic modalities of infective endocarditis

The term **complement** (C) is applied to a system of components present in the serum of man and animals. It consists of nine different proteins denoted C1–C9. The fraction C1 occurs in serum as calcium ion dependent complex, which on chelation with EDTA yields three protein subunits called C1q, C1r and C1s. Thus, C is made up of 11 different proteins. Though some of its components are stable, C as a whole is heat-labile undergoing spontaneous denaturation slowly at room temperature and in 30 minutes at 56°C. Serum deprived of C activity by heating it at 56°C for 30 minutes is said to be inactivated.

The amount of C present in the serum cannot be increased by immunization. It is, biologically, of considerable importance as an amplifier of immune reactions involving humoral antibodies and is believed to play an important role in the defence of the body against microbial infections. C does not bind to free antigen or antibody but only to antibody which has combined with its antigen. C binding site is located on the C_H2 domain of the Fc portion of IgM and IgG molecules only. These sites are not exposed when antibodies are in the uncombined state. However, after antibody combines with antigen the C binding site is exposed.

A single molecule of IgM can sensitize red cells to C lysis. On the other hand, sensitization by IgG requires that at least two molecules should be bound at adjacent sites on red cell surface. If IgG binding sites are too far apart, as in Rh system, C binding and lysis cannot occur.

C is normally present in the body in an inactive form but can be activated to form an enzyme cascade. The cascade is

a series of reactions in which the preceding components act as enzymes on the succeeding components cleaving them into dissimilar fragments. The larger fragments join the cascade and the smaller fragments are released which often possess biological effects which contribute to defence mechanism by:

- Initiating an inflammatory response.
- Causing the destruction of parasites, bacteria, virus-infected cells or red blood cells.
- Clearing dead cells and immune complexes.
- Detoxifying endotoxins.
- Effecting release of histamine from mast cells.

There are two **activation mechanisms** through which complement system executes its role. These are known as classical pathway and alternative or properdin pathway.

Classical pathway of complement activation

Activation of classical pathway of complement requires the presence of antibody, either IgM or IgG, bound to cell surface antigen or as an antigen–antibody immune complex. All the 11 proteins of the complement comprise the classical complement pathway. All are designated by C followed by the number of the component (complement's protein). Inactive components are described as C1, C2, C3 and so on. Activated forms are designated by placing a bar over the number, for example, C $\bar{2}$ represents activated C2 which is actually C2b.

The classical pathway (Fig. 12.1) is initiated when C1 interacts with the Fc portion of either cell-bound Ig (IgG or IgM) or immune complex. This interaction results in the sequential activation of C4, C2 and C3 and leads to the formation of complex cleaving enzymes. Activation of C5, C6, C7, C8 and C9 then completes the cascade and results in the formation of the **C5 to C9 membrane attack complex (MAC)**, which can lyse the cell. C1 is composed of one molecule of C1q, and two molecules each of C1r and C1s. C1q binds to Fc portion of the antibody molecule. Binding of this component of C1 causes a conformational change in the C1 complex that leads to the autoactivation of C1r.

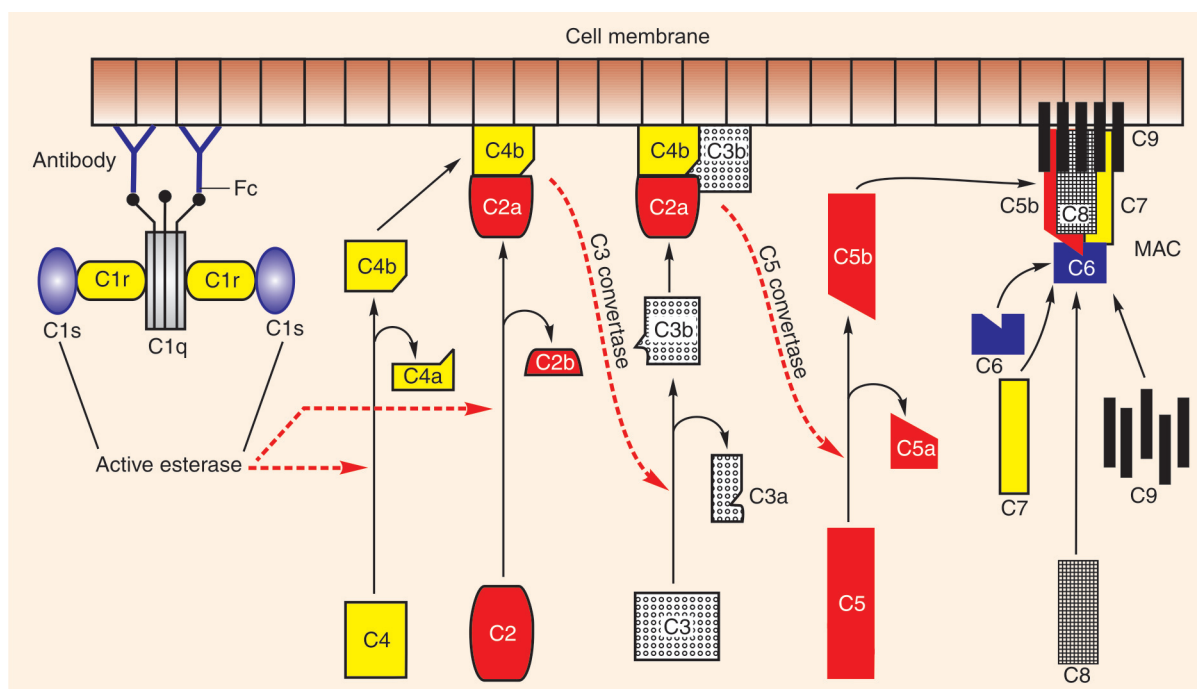


Fig. 12.1: Classical pathway of complement activation.

This then converts C1s into an active esterase that acts on C4 to produce C4a and a reactive C4b. C4a is released and less than 1% of the C4b becomes attached to the cell membrane. Unbound molecules of C4b are rapidly inactivated. C1s also cleaves C2 into two components—C2a, larger component, and C2b, smaller component. C2a attaches itself to membrane-bound C4b to form a new active protease, $C4b2a$, which is called C3 convertase since it can bind and cleave the next inactive complement component in the sequence, C3.

The newly formed C3 convertase, $C4b2a$ cleaves C3 into two fragments, C3a and C3b. The larger C3b fragment attaches to both the cell membrane and $C4b2a$ complex, while the smaller fragment, C3a, is released to the body fluids. C3a has chemotactic and anaphylatoxic properties. $C4b2a3b$ is termed C5 convertase. It cleaves C5 into two products, C5a and C5b. C5a which is a powerful chemotactant of neutrophils and monocytes and has anaphylatoxic activity is released when formed and C5b attaches to the cell membrane. The binding of C5b leads to the uncovering of a binding site for C6 and C7 on the molecule, producing a stable complex $C5b67$.

This trimolecular complex attaches to the membrane surface and enables C8 to join. C8 then binds several C9 molecules. About 10–18 protein units of C9 attach to $C5b678$ base to form a long hollow tube. This is a stable complex and is referred to as membrane attack complex (MAC). This creates a membrane pore or lesion, that is 100 Å in diameter leading to cell death. Pores in the cell membrane created by MAC may also permit degradative enzymes in the area to enter and destroy cellular organelles contributing to target cell death.

Alternative or properdin pathway of complement activation

This pathway does not require the presence of specific antibodies. C3 is the major component of C. In the classical pathway, activation of C3 is achieved by the C3 convertase ($C4b2a$). The activation of C3, without the prior participation of C1, 4 and 2, is known as the alternative or properdin pathway of complement activation. The overall result of this pathway is the same as that of classical pathway but the C3 and C5 convertases for alternative pathway are different from those of classical pathway. A wide range of chemically unrelated substances are known to activate alternative pathway. These (other than antigen–antibody complexes) include:

- Yeast cell walls.
- Bacterial endotoxins.
- Rabbit (not sheep) RBCs.
- Snake venom proteins.
- A protein termed ‘nephritic factor’ found in the serum of patients with diseases such as glomerulonephritis.

The fact that these products can activate the alternative pathway directly, without the need of antibody is of considerable importance because it allows for defence against infection prior to initiation of an immune response.

There are at least three normal serum proteins that, when activated together with C3, form a functional C3 convertase and a C5 convertase. These are factor B, factor D and properdin. These are normal serum proteins, and the alternative pathway is routinely being activated in the absence of any stimulus. In the absence of initiators, the initial complexes of the alternative pathway are rapidly destroyed. In the

presence of the initiators, such complexes are stabilized and complement is activated to form the MAC as in case of classical pathway.

Intrinsically, C3 undergoes a low level of hydrolysis of an internal thioester bond to generate C3b. Non-immune activators, such as repeating polysaccharide units or the lipopolysaccharide found on the cell walls of some microbes split up C3 into C3a and C3b. There are a wide variety of pathogens that can be recognized within minutes after they come in contact with plasma. Organisms sensitive to attack by the alternative pathway include bacteria, fungi, certain viruses, virus-infected cells, parasites and certain tumour cells. C3b, in the presence of Mg^{2+} , binds to these foreign surfaces and interacts with plasma protein factor B forming C3bB (Fig. 12.2).

The factor B portion of C3bB complex is split by factor D into two fragments, Ba and Bb. Ba is released during reaction and Bb remains bound to C3b forming C3bBb. The newly formed C3bBb is a C3 convertase of alternative pathway. C3bBb splits more C3 to C3a and C3b. The newly

formed C3b binds more factor B. This continues until the membrane surface is saturated with C3bBb. The result is opsonization of the cell or particle by neutrophils. The soluble C3a, that is released upon cleavage of C3, has chemotactic and anaphylatoxic activity, which can initiate an inflammatory response. C3 convertase (C3bBb) can bind additional C3b to produce C5 convertase (C3bBb3b). This activates terminal lytic complement sequence, C5 to C9.

Thus, activation of either classical or alternative pathway leads to the formation of C3 convertases (C4b2a or C3bBb). These cleave C3 into C3a and C3b. The latter combines with C3 convertases forming C5 convertases (C4b2a3b or C3bBb3b). These split C5 into C5a and C5b. C5b then binds C6, C7, C8 and several molecules of C9 to form MAC which initiates lysis of target cells in both pathways.

Control of complement system

It is clear that, with such enormous potential for the tissue destruction, there must be a strict control at almost every stage of the complement system. This control is exerted by proteins which inactivate key components either by binding to the active sites of the enzymes (e.g. C1 inhibitor) or by further cleaving active proteins to give inactive products (e.g. the cleavage of C3b by factor I).

C1 inhibitor: It is a glycoprotein present in the normal serum. It inhibits C1r and C1s by binding to active site.

Factor I: Control of C3b is necessary for the regulation of both the classical and alternative pathways. Factor I is an endopeptidase which cleaves C3b and C4b.

Factor H: It is a β -globulin present in normal serum. It regulates alternative pathway by binding to C3b. Once bound it exerts its control in two ways:

1. It prevents factor B from binding to C3b.
2. It dissociates Bb from the active complex C3bBb and makes the C3b susceptible to cleavage by factor I.

C4-binding protein: It is a normal human serum protein that binds tightly to C4b and enhances C4b degradation.

Factor P or properdin: Factor P or properdin which originally gave its name to the alternative pathway, binds to and stabilizes the alternative pathway C3 convertase when it is bound to activating surfaces. Properdin has two forms—native and activated. Native properdin, in plasma, can bind to C3bBb and activates it. Activated properdin can:

- Bind directly to bound and unbound C3b.
- Stabilize C3/C5 convertases.
- Induce further activation of complement components indirectly by extending the half-life of C3bBb.

Cobra venom factor (CoVF): It is a protein found in the venom of cobras. This protein activates complement and lyses erythrocytes. It can combine with factor B to form the complex CoVF-Bb, which is equivalent to C3/C5 convertase. This activates the terminal complement sequence C5 to C9 and lysis of erythrocytes. The venom seems to be resistant to the action of the inactivators of the alternative pathway, factors H and I.

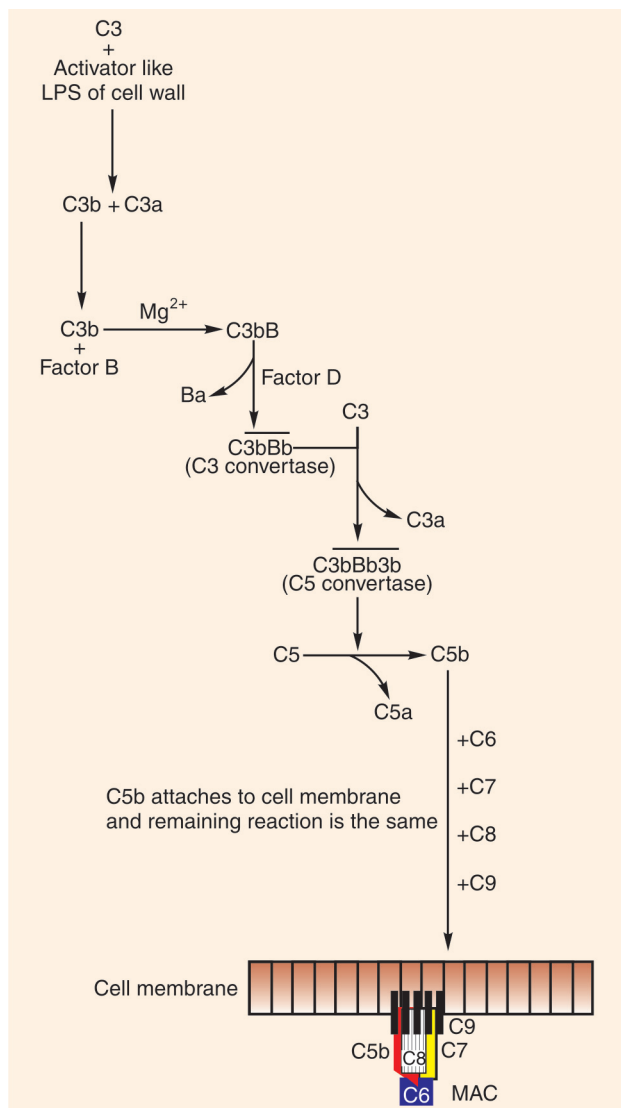


Fig. 12.2: Alternative pathway of complement activation.

Biological effects of complement

1. Complement mediates **immunological membrane damage**. This results in bacteriolysis and cytolysis. Different cells vary in their susceptibility to complement-mediated lysis. Gram-negative bacteria are generally sensitive to lysis, while Gram-positive are killed without lysis. Neutralization of certain viruses requires the participation of C, e.g. neutralization of herpesvirus by IgM antibody requires the binding of C1, C4 and possibly C3 too.
2. C fragments released during cascade reaction help in **amplifying the inflammatory response**. Proteolytic cleavage of C3 and C5, in either the classical or alternative pathway, generates two potent mediators of inflammation, C3a and C5a. Mast cells and basophils possess receptors for C3a and C5a. Binding of C3a or C5a to these receptors causes these cells to release histamine. This may lead to contraction of the uterus, trachea, arteries, atrium of the heart and intestines, and increased vascular permeability leading to oedema. C5a, in addition exerts a series of unique effects on white blood cells. These include:
 - Degranulation and lysosomal enzyme release.
 - Promotes adherence of granulocytes to the endothelium.
 - Induces chemotactic migration of granulocytes.

C5b67 is also chemotactic. C4a has weak anaphylatoxic activity. It is weakly spasmogenic and increases vascular permeability. Therefore, redness, pain, swelling and heat of inflammation is due to the action of C4a, C3a, C5a and histamine.
3. Phagocytes such as macrophages, monocytes and neutrophils possess surface receptors for C3b. If immune complexes have activated the complement system, the C3b bound to them facilitates their recognition and ingestion by these phagocytes. This facilitated phagocytosis is referred to as **opsonization**.
4. Complement participates in **type II (cytotoxic) and type III (immune complex) hypersensitivity** reactions. The destruction of erythrocytes, following incompatible blood transfusion is an example of type II hypersensitivity. Participation of C is required for the production of immune complex diseases such as serum sickness and Arthus reaction (type III hypersensitivity).
5. Several serum C components are lowered in many autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis. These may, therefore, be involved in the pathogenesis of autoimmune diseases. C plays a major role in the pathogenesis of autoimmune haemolytic anaemia, paroxysmal nocturnal haemoglobinuria and hereditary angioneurotic oedema.
6. C3 and C6 participate in coagulation process.
7. C bound to antigen-antibody complexes adheres to erythrocytes. This is known as **immune adherence**. It

contributes to defence against pathogenic microorganisms as such adherent particles are rapidly phagocytosed. C3 and C4 are necessary for immune adherence.

Quantitation of complement

Measurement of C levels in the serum can be accomplished by estimating the highest dilution of the serum lysing sheep erythrocytes sensitized with antierythrocytic antibody. The haemolytic unit of C (CH_{50}) may be defined as that amount of complement that lyses 50% of sensitized erythrocytes under defined standard conditions.

Biosynthesis of complement

Various C components are synthesized in different parts of the body, e.g. intestinal epithelium (C1), macrophages (C2, C4), spleen (C5, C8) and liver (C3, C6, C9). The site of synthesis of C7 is not known. The mechanism that controls synthesis of C is not known.

Complement deficiencies

Complete or partial deficiency of various components of C are known both in man and animals. These are associated with increased susceptibility of the host to various microbial infections (Table 12.1).

Table 12.1: Complement deficiency and associated diseases

Complement deficiency	Association with disease
Early C components (C1, C4, C2 and C3)	Immune and rheumatic disorders.
Late C components (C5, C6, C7 and C8)	Recurrent (<i>Neisseria</i>) infections.
C1q	Combined immune deficiency states.
C1r	Many infections and lupus-like symptoms.
C1s	Systemic lupus erythematosus.
C4	Lupus-like symptoms.
C2	Increased susceptibility to infections.
C3	Severe pyogenic infections.
C5	Recurrent infections of gastrointestinal tract.
C6, C7 and C8	Disseminated gonococcal infections and recurrent meningococcal meningitis.
C9	Not more susceptible to disease than other individuals in the general population.

Deficiency of C1 inhibitor is associated with hereditary angioneurotic oedema. Deficiency of C3b inactivator (factor I), factor D and properdin predisposes to recurrent infections.

KEY POINTS

- **The complement system**, a multicomponent triggered enzyme cascade, attracts phagocytic cells to the microbes which engulf them.
- Complement can be activated by classical and alternative pathways.
- The amount of complement present in the serum cannot be increased by immunization.
- Complement participates in type II and type III hypersensitivity.
- Several serum complement components are lowered in many autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis. They may, therefore, be involved in the pathogenesis of autoimmune diseases.
- Complement mediates immunological membrane damage.
- C fragments released during cascade reaction help in amplifying the inflammatory response.
- C3 and C4 mediate immune adherence.
- C3 and C6 participate in coagulation process.



Important Questions

1. Discuss briefly the biological effects of complement.
2. What is the sequence of events when the classical pathway of the complement system is activated?
3. Discuss the alternative pathway of complement.



Multiple Choice Questions

1. Classical pathway of the complement system is activated by:
 - (a) IgG.
 - (b) IgM.
 - (c) both of the above.
 - (d) none of the above.
2. Alternative pathway of the complement system is activated by:
 - (a) yeast cell walls.
 - (b) bacterial endotoxins.
 - (c) snake venom proteins.
 - (d) all of the above.
3. C1, C2 and C4 are components of:
 - (a) classical pathway of complement.
 - (b) alternative pathway of complement.
 - (c) both of the above.
 - (d) none of the above.
4. Factor B, factor D and properdin are components of:
 - (a) classical pathway of complement.
 - (b) alternative pathway of complement.
 - (c) both of the above.
 - (d) none of the above.
5. In classical pathway of complement activation the first component of complement which binds to Fc portion of antibody molecule is:
 - (a) C1q.
 - (b) C1r.
 - (c) C1s.
 - (d) C2.
6. Which of the following is C3 convertase of classical pathway of complement activation?
 - (a) $\overline{C4b2a}$
 - (b) $\overline{C3bBb}$
 - (c) $\overline{C4b2a3b}$
 - (d) $\overline{C3bBb3b}$
7. Which of the following is C3 convertase of alternative pathway of complement activation?
 - (a) $\overline{C4b2a}$
 - (b) $\overline{C3bBb}$
 - (c) $\overline{C4b2a3b}$
 - (d) $\overline{C3bBb3b}$
8. Which of the following is C5 convertase of classical pathway of complement activation?
 - (a) $\overline{C4b2a}$
 - (b) $\overline{C3bBb}$
 - (c) $\overline{C4b2a3b}$
 - (d) $\overline{C3bBb3b}$
9. Which of the following is C5 convertase of alternative pathway of complement activation?
 - (a) $\overline{C4b2b}$
 - (b) $\overline{C3bBb}$
 - (c) $\overline{C4b2b3b}$
 - (d) $\overline{C3bBb3b}$
10. A membrane attack complex that can lyse micro-organisms is produced by components of:
 - (a) complement.
 - (b) interferon alpha.
 - (c) interferon gamma.
 - (d) antibodies.



Answers

1. c 2. d 3. a 4. b 5. a 6. a 7. b 8. c 9. d 10. a

Antigen–Antibody Reactions

Competency achievement: The student after reading the chapter should be able to:

MI1.10(a): Describe the immunological mechanisms of hypersensitivity and discuss the laboratory methods used in detection of hypersensitivity.

MI1.10(b): Describe the immunological mechanisms of autoimmunity.

MI1.10(c): Describe immunological mechanisms in immunological disorders in immunodeficiency states.

MI1.11: Describe the immunological mechanisms of transplantation and tumour immunity.

MI2.1: Describe etiologic agents of rheumatic fever and their diagnosis.

When an antigen is mixed with its specific antibody, in the presence of electrolytes at a suitable temperature and pH, they combine with each other in an observable manner.

- **In the body**, they form the basis of:
 - Antibody-mediated immunity in infectious diseases.
 - Tissue injury in some types of hypersensitivity and autoimmune diseases.
- **In the laboratory**, they help in the diagnosis of:
 - Infectious diseases.
 - Non-infectious agents such as enzymes.

These reactions can be used for the detection and quantitation of either antigen or antibody.

Antigen–antibody reactions *in vitro* are known as *serological reactions*.

Advantages of serologic testing

- Detection of infectious agents that are either difficult to culture (e.g. human immunodeficiency viruses and hepatitis viruses) or impossible to culture, usually because of either their specific growth requirements or previous antimicrobial therapy that decreases the number of viable organisms.

- Detection of fungi and mycobacteria that take extremely long time to grow.
- Detection of microorganisms cultivation of which is significant risk to laboratory workers.

Disadvantages of serologic testing

- To measure the host response to an organism, 10–14 days must have passed after the onset of an infection.
- In case of hepatitis B virus and hepatitis C virus infections, weeks to months must have passed before antibody levels are detectable.
- Immunocompromized patients can have an antibody response that is either diminished or non-existent, impairing the ability to use any serologic procedure.
- Antibody detected may have been produced against another organism, and a **false-positive** test is observed.

CHARACTERISTICS OF ANTIGEN–ANTIBODY REACTIONS

- Antigen–antibody reaction is specific but cross-reactions may occur. This is due to antigenic similarity or relatedness.
- Antigen–antibody combination is firm but reversible.
- There is no denaturation of antigen or antibody during the reaction.
- Binding takes place on the surface. Therefore, surface antigens are more relevant.
- Entire molecules react. Therefore, when an antigenic determinant present on a large molecule or a carrier particle reacts with antibody, whole molecules or particles are agglutinated.

For better understanding of antigen–antibody reactions a few terms are defined below.

Affinity: Intensity of the attraction between an antibody-combining site and an antigenic determinant.

Avidity: Strength of the bond after the formation of antigen–antibody complexes.

Sensitivity: Ability of a test to identify correctly all those who have the disease, i.e. true positives. A 90% sensitivity

means that 90% of the diseased persons screened by the test will give a true positive and 10% a false negative result.

Specificity: Ability of a test to identify correctly all those who do not have the disease, i.e. true negatives. A 90% specificity means that 90% of non-diseased persons screened by the test will give a true negative and 10% a false positive result. In other words, 10% of non-diseased persons will be wrongly classified as diseased when they are not.

Stages

The reactions between antigens and antibodies occur in three steps:

1. **Primary stage:** The initial interaction between antigens and antibodies is rapid without any visible effect. The combination between antigen and antibody molecules occurs by weaker intermolecular forces such as van der Waals forces, ionic bonds and hydrogen binding, rather than by firmer covalent binding. This reaction is reversible. The primary reaction can be detected by a number of physical and chemical methods.
2. **Secondary stage:** The primary stage in most, but not all, instances is followed by secondary stage. This leads to demonstrable events such as precipitation, agglutination, lysis of cells, neutralization, complement fixation, immobilization of motile organisms and enhancement of phagocytosis.
3. **Tertiary stage:** Some antigen–antibody reactions occurring *in vivo* initiate chain reactions that lead to neutralization or destruction of injurious antigens, or to tissue damage. These are tertiary reactions and include humoral immunity against infectious diseases as well as clinical allergy and other immunological diseases.

METHODS USED TO DETECT AND QUANTITATE ANTIGEN AND ANTIBODY

A number of methods can be used to determine the presence or amount of antigens and antibodies. Measurement may be in terms of mass (e.g. mg nitrogen) or more commonly as units or titre.

- **Antibody titre** is the highest dilution of serum which gives an observable reaction with the antigen in a particular test.
- **Agglutination:** If the antigen is on the surface of particulate material, such as a bacterium or a red blood cell, the end result is a clumping of the cells or agglutination.
- **Precipitation:** A soluble antigen may form an antigen–antibody complex that becomes too large to stay in solution and the result is a precipitation.
- **Antitoxin:** An antibody may neutralize a bacterial toxin. Antibody neutralizing the toxin is known as antitoxin.
- **Neutralizing antibodies:** An antibody may neutralize viruses so that they cannot infect susceptible cells. These are known as neutralizing antibodies.
- **Opsonins:** Antibodies which react with bacterial cells to make them more easily phagocytosed by phagocytes are known as opsonins.

However, multiplicity of antibody names does not mean that different types of antibodies exist for each reaction. Thus, if a soluble antigen is mixed with specific antibody it leads to precipitation and when the same antigen is coated on particles such as polystyrene beads, the same specific antibody will agglutinate the beads or enhance their phagocytosis. With this in mind, a number of antigen–antibody reactions used to detect the presence of antigen or antibody in clinical specimens are described below.

Precipitation reactions

Precipitation

When a soluble antigen is mixed with its specific antibody in the presence of electrolytes at a suitable temperature and pH, the antigen–antibody complex forms an insoluble precipitate. This precipitate usually settles down at the bottom of the tube. Precipitation can take place in liquid media and in gels such as agar, agarose and polyacrylamide. The process of precipitation can be hastened by electrically driving the antigen and antibody.

Flocculation

When, instead of sedimenting, the precipitate remains suspended as floccules, the reaction is called flocculation.

Zone phenomenon

If a series (10–12) of tubes is set up (Fig. 13.1), each containing a constant amount of antiserum, and increasing amounts of antigen are added to the tubes in the row, precipitation will be found to occur most rapidly and abundantly in one of the middle tubes, in which antigen and antibody are in optimal or equivalent proportion. In the preceding tubes, in which the antibody is in excess, and in the later tubes, in which the antigen is in excess, the precipitation will be weak or absent. Therefore, the amount of precipitation will be seen to increase along the row, reaching a maximum and then falling off with higher antigen concentration.

If the amounts of precipitate in different tubes are plotted on a graph, the resulting curve will have **three phases**—an ascending part (**prozone** or **zone of antibody excess**), a peak (**zone of equivalence**), and a descending part (**postzone** or **zone of antigen excess**). This is called **zone phenomenon**. Assay of supernatant solution will show that those tubes containing too little antigen still contain free antibody and in the tubes with antigen excess, little precipitate forms, although soluble immune complexes and free antigens are present in the supernatant fluid. Only in tubes of maximum precipitation is all antibody removed from solution. *The prozone is of importance in clinical serology, as sera rich in antibody may sometimes give a false negative result, unless several dilutions are tested.*

If immune complexes form in serum, monocytes, neutrophils and eosinophils attempt to remove them. Complexes formed at equivalence or antibody excess are easily removed. However, small, soluble complexes formed in antigen excess are more difficult to remove. These might gain entrance to

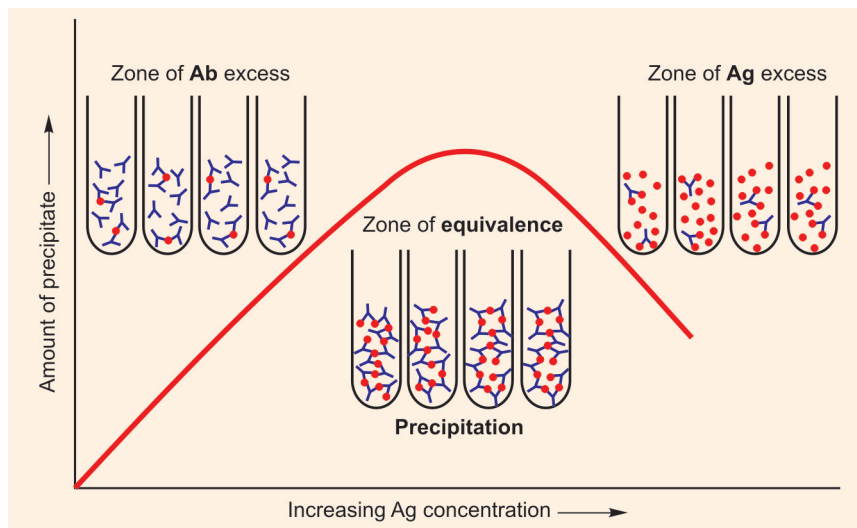


Fig. 13.1: Zone phenomenon.

tissues, such as glomeruli of kidneys or become deposited within vessel walls causing varying degree of damage.

Mechanism of precipitation

To explain the mechanism of precipitation, Marrack, 1934 proposed a **lattice hypothesis**. Multivalent antigens combine with bivalent antibodies in varying proportions, depending upon the antigens and antibodies in the reacting mixture. When the antigens and antibodies are in optimal proportion a large lattice is formed consisting of alternating antigen and antibody molecules. Therefore, most abundant precipitation occurs when both antigens and antibodies are in optimal proportion. In antibody excess each antigen or two combine with an independent molecule of antibody. Therefore, the lattice does not enlarge. Similarly, in antigen excess the lattice does not enlarge (Fig. 13.2).

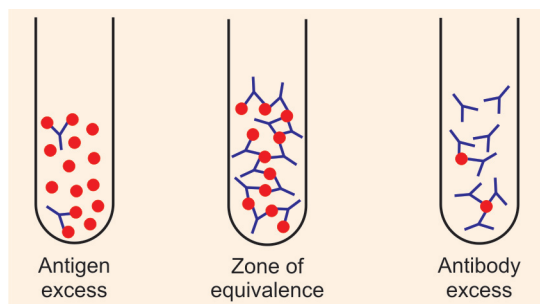


Fig. 13.2: Lattice formation.

Applications of precipitation and flocculation reactions

- The precipitation test may be carried out for both qualitative and quantitative determination of both antigen and antibody. It is a very sensitive test for detecting antigens and is relatively less sensitive for detection of antibodies. The precipitation test is capable of detecting as little antigen proteins as 1 µg.

- It can be used for identification of blood and seminal stains and food adulterants.

Some of the precipitation and flocculation tests which have application in diagnostic bacteriology are as under.

Ring test

This test is done by layering antigen solution over a column of antiserum in a capillary tube. After a short while a ring of precipitate forms at the interface. *Typing of streptococci and pneumococci*, *C-reactive protein test* and *Ascoli's thermo-precipitin test* for the diagnosis of anthrax, are some of the uses of ring test. This technique is also used in *detection of adulteration of foodstuffs*.

Slide test

This is an example of flocculation test. When a drop each of antigen and antiserum are placed on a slide and mixed by shaking, floccules appear. *VDRL*, a most widely used test for the diagnosis of syphilis, is an example of slide flocculation test.

Tube test

Flocculation test can be carried out in the tubes also. *Kahn test* for the diagnosis of syphilis, and *standardization of toxins and toxoids* are examples of tube flocculation tests.

Immunodiffusion (precipitation in gel)

When an antibody and its antigen are placed in an agar gel they diffuse towards each other and form an opaque band of precipitation at the junction of their diffusion front. Precipitation in gel has several advantages over precipitation in liquid medium:

- The reaction appears as a distinct band of precipitation which can be stained for better visibility and preservation.
- Since each antigen–antibody reaction gives rise to one line of precipitation, therefore, different number of antigens in a mixture can be detected.
- This technique also indicates *identity*, *cross reaction* and *non-identity* between different antigens.

Types of immunodiffusion tests

1. Single diffusion in one dimension (Oudin procedure)

Antibody is incorporated in agar gel in a test tube. Antigen solution is then layered over it. The antigen diffuses downwards and wherever it reaches in optimum concentration with antibody a line of precipitation is formed (Fig. 13.3A). As more antigen diffuses, the line of precipitation moves downwards. Number of lines of precipitation indicates the number of antigens and antibodies present.

2. Double diffusion in one dimension (Oakley-Fulthorpe procedure)

Antibody is incorporated in agar gel in a test tube. Above this is placed a column of plain agar which in turn is overlaid with antigen, either as liquid or incorporated into agar (Fig. 13.3B). Antigen and antibody diffuse (double diffusion) towards each other (in one dimension) through the intervening column of plain agar and form a band of precipitation where they meet in optimum concentration.

3. Single diffusion in two dimensions (radial immunodiffusion)

This method is used to quantitate the amount of a specific antigen present in a sample and can be used for many antigens. The most widely used diagnostic application of this procedure is to measure the amount of various Ig classes (IgG, IgA, IgM, IgD and IgE) in patient serum.

Here monospecific antiserum (antiserum containing only antibody against the antigen which is to be assayed) is incorporated in agar gel. It is poured on a glass slide or a

Petri dish and a number of wells are punched into it, and different dilutions of the antigen are placed into various wells. As the antigen diffuses from the well, a ring of precipitate forms at that position where antigen and antibody are in optimal proportions. Larger the concentration of antigen, the farther it diffuses to be in optimal proportions with the antibody incorporated in the gel. Therefore, the diameter of the ring gives the estimate of the concentration of the antigen (Fig. 13.3C).

Using known concentrations of the antigen in question, one can prepare a standard curve by plotting the diameter of the precipitin ring versus antigen concentration. With this standard plot, one needs only to measure the diameter of the precipitin ring formed with the unknown antigen to calculate its concentration. Radial immunodiffusion is used for the laboratory diagnosis of multiple myeloma or agammaglobulinaemia.

4. Double diffusion in two dimensions (Ouchterlony procedure)

Agar is poured on the slide and wells, usually seven, are punched in it using a template. The known antiserum is placed in the central well and different antigens in surrounding wells. One of these contains known positive antigen. It acts as a positive control. This technique is also useful for comparing different antigens for the presence of identical or cross reacting components (Fig. 13.3D).

1. **Reaction of identity:** If two precipitin bands fuse completely, the pattern is termed reaction of identity. It indicates that the antigens in the adjoining wells are identical.

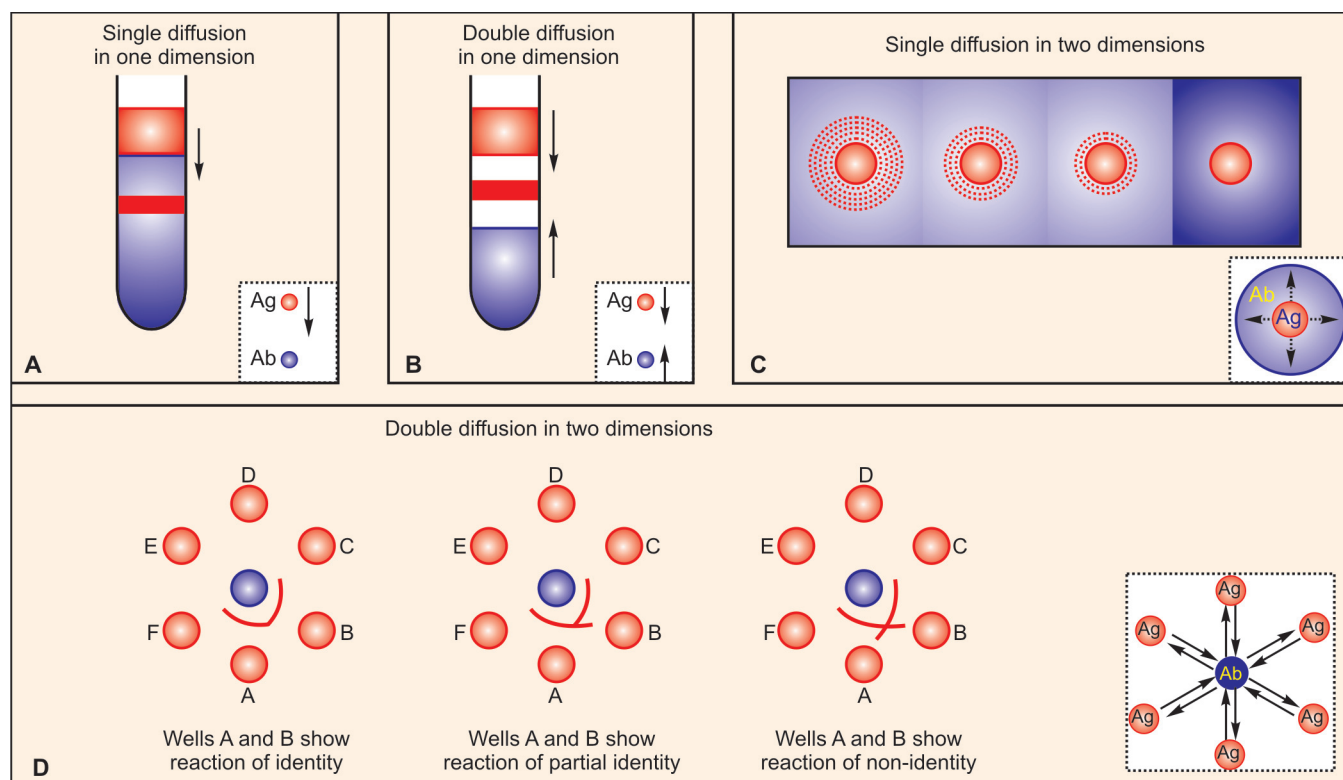


Fig. 13.3: Immunodiffusion tests.

2. **Reaction of partial identity:** If the antigens in the two adjacent wells are cross reacting (partial identity), the precipitation bands fuse but form a spur-like projection. This is known as reaction of partial identity.
3. **Reaction of non-identity:** If unrelated antigens are placed in adjacent wells, they diffuse towards central well containing antibodies for both, the two precipitin bands form independently and cross each other. This is known as reaction of non-identity.

A special variety of double diffusion in two dimensions is the **Elek's test** for toxigenicity of diphtheria bacilli (see Chapter 25).

5. Immunelectrophoresis

Immunelectrophoresis combines electrophoresis and immunodiffusion (immune precipitation in gel). This method can be used for analyzing complex antigens in biological fluids. A glass slide is covered with molten agar or agarose. A well for antigen and a trough for antiserum is cut on it (Fig. 13.4). Antigen well is filled with antigen mixture (human serum). The slide is then placed in an electric field for about an hour to allow for the electrophoretic migration of various antigens. Different antigens will migrate at

different rates or even in different directions, depending upon their size and charge and the conditions of electrophoresis.

After the completion of electrophoresis, antiserum trough is filled with appropriate antiserum (antiserum to whole human serum). Antigens and antibodies diffuse towards each other, resulting in the formation of precipitin bands, for individual antigens and antibodies, whenever they are both in zones of optimal proportions, in 18–24 hours. Because immunelectrophoresis uses electric charge in addition to diffusion, it is more likely to separate antigens than is simple diffusion alone. By this method, over 30 different antigens can be identified in human serum. This technique is useful for detection of normal and abnormal serum proteins.

6. Electroimmunodiffusion

Immunodiffusion is a slow process. The development of precipitin lines can be speeded up by electrically driving antigens and antibodies in a gel, rather than simply allowing them to come in contact by diffusion. Of these, one dimensional double electroimmunodiffusion and one dimensional single electroimmunodiffusion are used frequently in the clinical laboratory.

I. One dimensional double electroimmunodiffusion (counterimmunoelectrophoresis or CIE): This method can be used for those antigens and antibodies that migrate in opposite directions in electric field. The wells are punched about 1 cm apart in an agar slab on a glass plate. Antigen and antibody solutions are placed in wells towards cathode and anode sides, respectively. Electric field is then applied electrophoresing both antigens and antibodies from separate wells. The antigen migrates towards antibody, and antibody migrates towards antigen. A precipitin band is formed, in between the two wells, where they meet in optimum proportions (Fig. 13.5). This method has several advantages over simple diffusion in agar:

- The electrophoresis focuses the reactants into a small area allowing the detection of small quantities of antigens and antibodies. Therefore, **it is 10 times more sensitive than simple diffusion in agar.**
- It is a rapid assay. Precipitin bands may form in just 30 minutes.

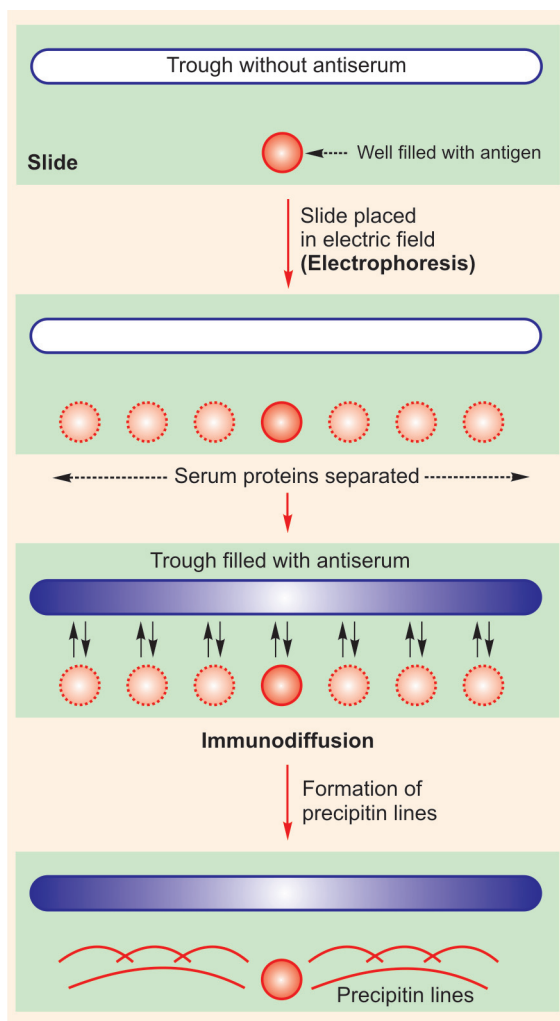


Fig. 13.4: Immunelectrophoresis.

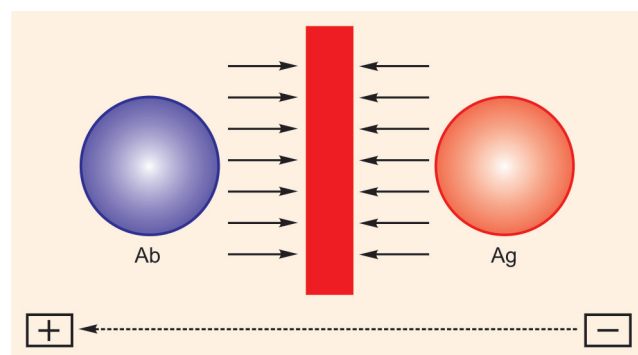


Fig. 13.5: Counterimmunoelectrophoresis.

This method is used for detection of various antigens such as:

- Hepatitis B surface antigen (HBsAg) and alpha-fetoprotein in serum;
- Meningococcal and cryptococcal antigens in CSF; and
- Anti-DNA antibody in the serum of patients with several autoimmune disorders.

II. One dimensional single electroimmunodiffusion (rocket electrophoresis): As in case of radial immunodiffusion, wells are cut in an agarose gel slab on a glass plate. Agarose contains the antiserum to the antigen of interest. The antigen, in increasing concentrations, is placed in wells. The antigen is then electrophoresed into the agarose containing antibody that does not migrate. The pattern of immunoprecipitation resembles a rocket (hence the name), since precipitation occurs along the moving boundary of antigen, as it migrates into the agarose (Fig. 13.6). The height (distance from the antigen well to the top of the precipitin band) is proportional to the antigen concentration. The main application of this technique, therefore, is for quantitative estimation of antigen.

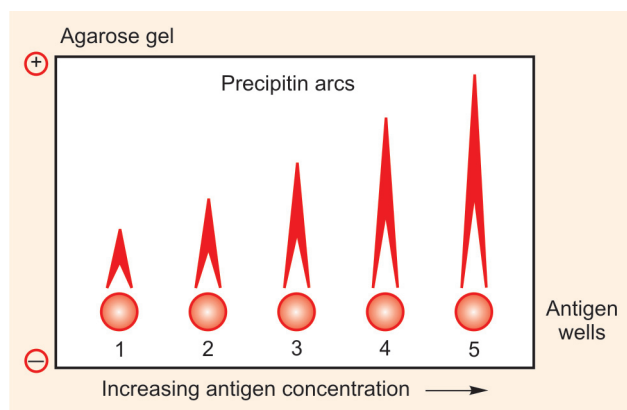


Fig. 13.6: Rocket electrophoresis.

Agglutination reactions

When a particulate antigen or an antigen present on the surface of a cell (red cell or bacterium) or an inorganic particle (e.g. polystyrene latex coated with antigen) is mixed with its antibody in the presence of electrolytes at a suitable temperature and pH, the particles are clumped or agglutinated. This reaction is analogous to precipitation reaction, in that antibody merely acts as a bridge to form a lattice network of antibody and cells or inorganic particles coated with antigen. Because cells or particles are much larger than soluble antigen, therefore, they aggregate into clumps. Agglutination reaction is more sensitive than precipitation for detection of antibodies.

Prozone phenomenon

False negative agglutination reactions can occur with some antisera in antibody excess (first few dilutions). This is known as prozone phenomenon. Unagglutinated cells in prozone actually have antibody molecules adsorbed on their surface, with both sites of bivalent antibody attached to the same cell resulting into poor or no lattice formation.

Blocking antibodies

Occasionally, antibodies (e.g. anti-Rh and anti-*Brucella*) are formed that react with the antigenic determinants on a cell but do not cause agglutination. Such antibodies are called blocking antibodies, because they inhibit agglutination by complete antibody added subsequently.

Applications of agglutination reactions

Slide agglutination

A drop of saline is placed on a clean glass slide and a small amount of culture from a solid medium is emulsified in it by means of inoculating loop. It is then examined through a hand lens or low-power microscope that the suspension is even and bacteria are not autoagglutinable. Then with a platinum loop a drop of specific antiserum is placed on the slide near the bacterial suspension. The serum and the bacterial suspension are then mixed and examined with naked eye or with hand lens or under low-power microscope for the evidence of agglutination within a minute. Slide agglutination test is rapid and convenient, but in order to obtain rapid agglutination serum is used undiluted or in low dilutions.

Uses:

- Identification of bacterial isolates (e.g. *Salmonella* serotypes, *Shigella* spp. and *Vibrio cholerae*) from clinical specimens. This method is practicable only when clumping of organisms occurs instantaneously or within a minute because clumping occurring after a minute may be due to drying of the fluid.
- Blood grouping and cross matching.

Tube agglutination

This is done in round-bottomed test tubes or perspex plates with round-bottomed wells. A fixed volume of a particulate antigen suspension is added to an equal volume of serial dilutions of the patient serum in test tubes or perspex plates. Following several hours of incubation at 37°C, agglutination is seen at the bottom of the tubes. **The titre of the serum is given as the reciprocal of the highest dilution that causes agglutination.** Thus, the serum that agglutinates at a dilution of 1:256 is reported to have a titre of 256 and if the test has been carried out in 1 ml volumes, the titre of the serum is 256 units/ml of serum.

Uses:

Serological diagnosis of:

- Enteric fever (Widal test)
- Brucellosis
- Typhus fever (Weil-Felix reaction)
- *Streptococcus* MG agglutination
- Cold agglutination
- Paul-Bunnell test

In the **Widal test** used for the diagnosis of enteric fever, two types of antigens are used—the flagellar (H) antigen and somatic (O) antigen. H antigen is a formalised suspension of the organisms which on combination with antibody, forms large, loose and fluffy clumps resembling wisps of cotton-

wool. For H agglutination conical (Dreyer's) tubes are used. O antigen is prepared by treating the bacterial suspension with alcohol. On combination with antibody it forms fine granular deposit resembling chalk powder at the base of round-bottomed (Felix) tubes, whereas, negative reaction shows a compact button-like deposit (Fig. 13.7).

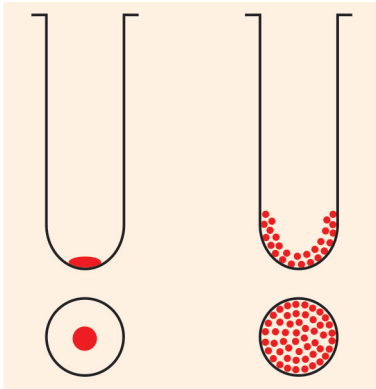


Fig. 13.7: Tube agglutination test.

Heterophile agglutination

Weil-Felix reaction for serodiagnosis of typhus fever and **Streptococcus MG agglutination** for the diagnosis of primary atypical pneumonia are the examples of **heterophile agglutination test**. Red blood cells are used as antigens in **cold agglutination** and **Paul-Bunnell test**. IgM antibodies capable of agglutinating human red cells at 0–4°C (cold agglutinins) are sometimes found in certain human diseases including primary atypical pneumonia, malaria, trypanosomiasis and acquired haemolytic anaemia. Sera of the patients suffering from infectious mononucleosis agglutinate sheep RBCs (see Chapter 10).

Antiglobulin (Coombs') test

Anti-Rh antibodies are of IgG type, but they normally do not agglutinate Rh-positive RBCs (incomplete antibodies). The inability of these antibodies to agglutinate is perhaps due to the presence of insufficient antigenic determinants on the RBCs to permit the antibody to overcome the normal electrostatic repulsion that exists among RBCs. When sera containing incomplete anti-Rh antibodies are mixed with Rh-positive red cells, the antibody coats the surface of erythrocytes but they are not agglutinated. When such antibody-coated erythrocytes are washed to free all unattached protein and are treated with antihuman gamma-globulin (**antiglobulin or Coombs' serum**), the cells are agglutinated.

Coombs' test is of two types—direct and indirect.

1. **Direct Coombs' test:** The sensitization of erythrocytes with incomplete antibodies takes place *in vivo* as in case of haemolytic disease of newborn due to Rh incompatibility. Therefore, when washed RBCs from such patient are mixed with antiglobulin or Coombs' serum, agglutination takes place.

2. **Indirect Coombs' test:** The sensitization of RBCs with incomplete antibodies is performed *in vitro*. Rh-positive RBCs are mixed with the serum to be tested for Rh-antibodies and then after a short incubation and washing, antiglobulin or Coombs' serum is added. If the test serum contained anti-Rh antibodies, agglutination will take place.

Uses of Coombs' test

- Detection of anti-Rh antibodies
- Demonstration of non-agglutinating antibodies in brucellosis

Passive (indirect) agglutination

A precipitation reaction can be converted into agglutination reaction by coating soluble antigen onto the surface of carrier particles such as RBCs, latex, bentonite and gelatin particles. Such test is more convenient and more sensitive for detection of antibodies. Most polysaccharide and lipopolysaccharide antigens may be adsorbed by simple mixing with the cells. For adsorption of protein antigens, tanned red cells are used. Following are some of the examples of passive agglutination.

- In rheumatoid arthritis, **RA factor** (an antigammaglobulin autoantibody) appears in the serum of the patient. It acts as an antibody to human IgG. Latex polystyrene beads coated with denatured human IgG when mixed with patient serum leads to agglutination of latex polystyrene beads.
- Latex particles coated with antibodies to meningococci, *Haemophilus influenzae* type b and pneumococci can be used to detect corresponding antigens in cases of **pyogenic meningitis**.
- Latex agglutination tests are also widely used for detection of hepatitis B, antistreptolysin O, C-reactive protein, human chorionic gonadotropin hormone and many other antigens.
- One of the most widely used passive agglutination tests employing erythrocytes is **Treponema pallidum haemagglutination (TPHA)** for serological diagnosis of treponemal infection.
- For the detection of **anti-HIV antibodies**, gelatin particles can be sensitized (coated) with inactivated HIV antigen. When these sensitized particles are mixed with the patient serum or plasma these particles are agglutinated if the anti-HIV antibodies are present in the sample. The test procedure is extremely simple using a microtitre technique and is particularly suitable for mass screening of specimens. The test is time-saving and results are readable by the naked eye after about 2 hours.

When, instead of antigen, antibody is adsorbed on the carrier particles in tests for estimation of antigens, the technique is known as **reversed passive agglutination**.

Coagglutination

This is based upon the principle that most strains of *Staphylococcus aureus* (especially Cowan strain I) possess protein A on their surface. Protein A binds IgG molecules, non-specifically, through Fc region leaving specific Fab sites free to combine with specific antigen. When suspension of such sensitized staphylococcal cells is treated with

homologous (test) antigen, the antigen combines with free Fab sites of IgG attached to staphylococcal cells leading to visible clumping of staphylococci within 2 minutes. This is known as coagglutination (COA).

COA test can be used for detecting the presence of bacterial antigens in serum, urine and CSF. For example, typhoid bacillus antigen is consistently present in the blood in the early phase of disease, and also in the urine of the patients. This antigen can be detected by COA test. Similarly, meningococcal, pneumococcal and *Haemophilus* antigens can be detected by COA test in the CSF. Identification of *Neisseria gonorrhoeae* and serogrouping of β -haemolytic streptococci A, B, C, D and G can also be carried out by COA test.

Complement fixation test (CFT)

The ability of antigen–antibody complexes to fix complement is made use of in complement fixation test (CFT). This is a very versatile and sensitive test for both antibody and antigen detection. This can detect as little as 0.04 μg of antibody nitrogen and 0.1 μg of antigen. CFTs include **Wassermann reaction** and **Reiter protein complement fixation test (RPCFT)** for the serodiagnosis of syphilis. Similarly, CFTs for the identification of various viral antigens are also available.

In most of the cases fixation of complement with antigen–antibody complex causes in itself no visible effect. Therefore, it is necessary to use an indicator system consisting of sheep red cells coated with anti-sheep red cell antibody. Complement lyses antibody coated red cells. CFT, therefore, is performed in two stages.

Stage 1: Test serum (for the detection of antibody) and the antigen are mixed in the presence of carefully measured amount of complement and then incubated at 37°C for 1 hour. If the patient serum had specific antibody to the test antigen, complement would be fixed by the test system and would be unavailable to lyse the erythrocytes.

Stage 2: Indicator system, antibody-coated sheep red cells, is added to determine whether the complement has been fixed in stage 1 reaction or not. If the patient serum did not have specific antibody to the test antigen, complement would be fixed by the indicator system, resulting in erythrocyte lysis. Therefore, a positive CFT is indicated by absence of lysis of red cells whilst a negative test, with unused complement, is shown by lysis of the red cells (Fig. 13.8).

The antigen in this test may be soluble or particulate. Prior to commencement of test the serum should be inactivated by heating it at 56°C for 30 minutes to destroy any complement activity serum may have and also to remove some non-specific inhibitors of complement. The source of complement for laboratory use is guinea pig serum. Guinea pig serum is first titrated for complement activity. One unit or **minimum haemolytic dose (MHD) of complement** is the highest dilution of guinea pig serum that lyses one unit volume of washed sheep RBCs in the presence of excess of haemolysin (amboceptor) within a fixed time (usually 30–60 minutes) at 37°C. Similarly, amboceptor should also be

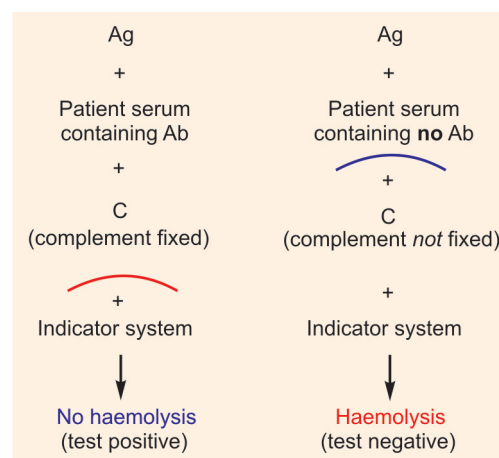


Fig. 13.8: Complement fixation test.

titrated. **One MHD of haemolysin** may be defined as highest dilution of the serum that lyses one unit volume of washed sheep RBCs in the presence of excess complement within a fixed time (usually 30–60 minutes) at 37°C.

Other tests employing complement

Immune adherence

Some bacteria, like *Vibrio cholerae* and *Treponema pallidum*, react with specific antibody in the presence of complement and particulate material such as erythrocytes or platelets; the bacteria are aggregated and adhere to the cells. This is known as immune adherence. **Adherence occurs through the activated C3b component of complement.**

Treponema pallidum immobilization test

The test serum is incubated anaerobically with a suspension of live treponemes and complement. If antibodies are present, the treponemes will be found to be immobilized.

Cytolytic or cytotoxic tests

When *V. cholerae* is mixed with its antibody in the presence of complement, the bacterium is killed and lysed.

Neutralization tests

These are of two types—virus neutralization tests and toxin neutralization tests.

Virus neutralization tests

Neutralization of viruses by their antibodies in a patient serum may be quantitated by their ability to reduce the infectivity of a stock virus preparation. Neutralization probably occurs because antibody binds to the viral particles and blocks subsequent attachment of the virus to receptor sites on target cells. The test serum is diluted serially, incubated with a known amount of virus and the mixture is then added to indicator systems—animals, embryonated hen's egg and tissue culture. The highest dilution of serum ablating infectivity in 50% of virus-serum mixtures tested is taken as the titre. Neutralization of bacteriophages can be demonstrated by plaque inhibition test. When bacteriophages are seeded in appropriate dilution

on lawn cultures, plaques of lysis are produced. Specific antiphage serum inhibits plaque formation.

Toxin neutralization tests

Bacterial exotoxins are highly antigenic and their activity may be completely neutralized by appropriate concentrations of specific antibody. Antibody to bacterial exotoxin is usually referred to as antitoxin. Bacterial endotoxins are poorly antigenic and their toxicity is not neutralized by antisera.

In vivo tests

- The neutralizing capacity of an antitoxin can be assayed by neutralization test in which mixture of toxin and antitoxin is injected into a susceptible animal and the least amount of antitoxin that prevents death or disease in the animal is estimated.
- In case of diphtheria toxin, which in small doses causes cutaneous reaction, neutralization test can be carried out on the human skin by Schick test. It is based on the ability of circulating antitoxin to neutralize the diphtheria toxin given intradermally. Neutralization (no reaction) indicates immunity, and erythema and induration indicates susceptibility to diphtheria.

In vitro tests

If a toxin has a demonstrable *in vitro* effect, this effect can be neutralized by specific antitoxin.

- For example, **antistreptolysin O**, present in the serum of the patient suffering from *Streptococcus pyogenes* infection, neutralizes the haemolytic activity of the streptococcal O haemolysin.
- Another example of *in vitro* toxin–antitoxin neutralization is **Nagler's reaction**. *Clostridium perfringens* produces α -toxin which is a phospholipase (lecithinase-C). This produces opalescence in serum or egg yolk media. This reaction is specifically neutralized by the antitoxin.

Opsonization

A substance, such as complement or antibody, that can bind to the surface of a cell or a particle, making it more readily phagocytosed is known as opsonin. Enhanced complement-mediated phagocytosis can occur either in the presence or absence of antibody. Phagocytes such as macrophages, monocytes and neutrophils possess surface receptors (CR1) for C3b and Fc receptors for antibody. If immune complexes have activated the complement system then Fc and CR1 receptors, present on the phagocyte, bind Fc region of antibody and C3b bound on immune complexes, respectively, thus facilitating their phagocytosis. This facilitated phagocytosis by antibody and complement is known as **immune opsonization** (Fig. 13.9).

In contrast, **non-immune opsonization** requires only C3b (opsonin) for opsonization. Bacteria in the blood stream can activate the alternative pathway and generate C3b, which coats the bacteria. C3b binds to CR1 receptors present on the phagocytes, thus facilitating their phagocytosis. Viruses, soluble immune complexes and tumour cells are also opsonized and removed by the same mechanism (Fig. 13.9).

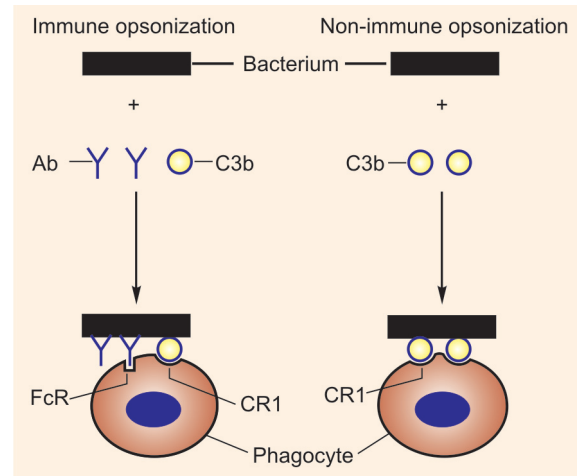


Fig. 13.9: Opsonization.

Immunofluorescence

Fluorescent dyes absorb invisible UV light between 290–495 nm and emit visible longer wavelength (525 nm) green light. Therefore, if microorganisms or tissue cells are stained with a fluorescent dye and examined under the microscope with UV light instead of visible light they are seen as bright objects against a dark background. This principle is used in fluorescence microscopy. Coons and his colleagues (1942) showed that fluorescent dyes, such as fluorescein isothiocyanate (FITC), can be conjugated to antibodies (without affecting their specificity) permitting their ready detection, when attached to an antigen associated with a cell. Immunofluorescence (IF) is now used extensively to detect:

- Tissue antigens.
- Antibodies to tissues including autoantibodies.
- The antigens of infecting organisms in the body.
- Antigen–antibody complexes.

It is more sensitive than precipitation and complement fixation test. Fluorescence can be observed under a fluorescence microscope (FM), which contains a high intensity UV light source (mercury lamp) instead of visible light. Two types of filters are fitted in the FM:

1. Primary filter: It is fitted close to the lamp. This ensures the maximum emission of radiation (UV light) of the required wavelengths.
2. Secondary filter: It is placed in the eyepiece to cut out UV rays which might damage the observer's eye.

Fluorescence-staining techniques are of two types—direct and indirect (Fig. 13.10).

Direct IF

This consists of bringing fluorescein-tagged antibodies in contact with antigens (bacteria, viruses and other antigens) fixed on a slide (e.g. in the form of a tissue section or a smear of an organism), allowing them to react, washing off excess antibody and examining under FM. The site of union of the labelled antibody with its antigen can be seen by the apple-green fluorescent areas on the slide. Direct IF is

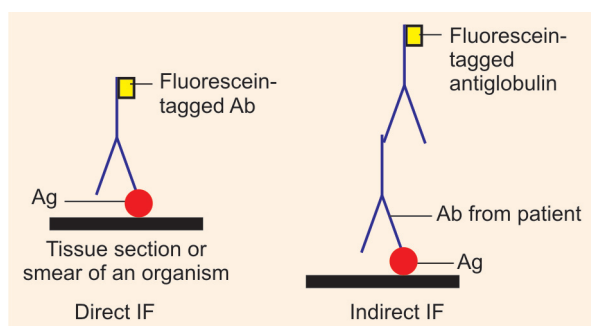


Fig. 13.10: Immunofluorescence.

routinely used as a sensitive method of diagnosing rabies, by detection of **rabies virus antigens** in brain smears. A **disadvantage of this method is that separate fluorescent conjugates have to be prepared against each antigen to be tested.**

Indirect IF

This method can be used for detection of specific antibodies in sera or other body fluids and also for identifying antigens. The disadvantage of direct IF, mentioned above, is overcome by this method. An example of this method is the **fluorescent treponemal antibody test for the diagnosis of syphilis**. Here a drop of the patient serum is placed on a smear of *T. pallidum* on a slide and after incubation, the slide is washed well to remove all free serum, leaving behind only antibody, if present, coated on the surface of the treponemes. Whether or not the patient serum contains antibodies to *T. pallidum* is shown by means of a fluorescein-tagged antihuman gamma-globulin (antiglobulin).

If patient serum contains antitreponemal antibodies fluorescein-tagged antiglobulin will react with it. After washing away all the unbound fluorescent conjugate, when the slide is examined under FM the treponemes will be seen as bright objects against a dark background. If the patient serum is negative for antitreponemal antibodies, there will be no antibody coating on the treponemes and, therefore, they will not take up the fluorescent conjugate. Therefore, they will not fluoresce. **The advantage of this technique is that a single antihuman gammaglobulin fluorescent conjugate can be employed for detecting human antibody to any antigen.** Indirect IF is also a convenient method for detecting autoantibodies that have bound to membrane antigens, *in vivo*.

The direct method is simple and rapid to perform with fewer non-specific reactions, however, it is less sensitive. The indirect method is more sensitive and gives brighter fluorescence, however, due to increased cross reactivity it is less specific.

Radioimmunoassay (RIA)

RIA is a very sensitive and specific method. It involves the use of either antiserum or more usually antigen labelled with ^{125}I . The amount of radioactive label bound to antigen-antibody complex can be measured, and hence the

concentration of antigen or antibody in a specimen can be determined. RIA permits measurement of analytes up to picogram (10^{-12} gram) quantities. It has been used to measure a variety of serum proteins including Igs, hormones, drugs, tumour markers and viral antigens. RIA is based on the competition of radiolabelled, known antigen and unlabelled, unknown antigen for limited binding sites on known specific antibody in solution or attached to a plate or tube (solid phase).

To establish an assay, antibody must be produced against the known antigen and the antigen radiolabelled, e.g. with ^{125}I . A standard curve is established by adding fixed amounts of radiolabelled antigen, specific antibody and increasing concentrations of known unlabelled antigen to a series of tubes or wells in a plastic microtitre plate. Following incubation, the amount of labelled antigen bound to antibody is determined after separation from free labelled antigen in a gamma spectrometer. If a test serum contains antigen for the antibody, then there is competition with labelled antigen. This appears as reduction in the amount of labelled antigen bound in the complex. The concentration of the antigen in the sample can be determined from the standard curve.

Enzyme immunoassay (EIA)

Enzyme immunoassay is an important immunological method for detecting and measuring antigens or antibodies. It is based on the same principle as that of radioimmunoassay. The key difference is that for enzyme immunoassays the antigen or antibody is conjugated to an enzyme rather than a radioactive isotope. The enzyme is then detected by its ability to convert a colourless substance to a coloured one. Obviously the method requires that, in the enzyme immunoglobulin conjugate, the enzyme retains its enzymatic activity and the antigen or antibody its immunological activity.

Enzyme immunoassays have become very popular in view of their high sensitivity, safety, economy and the simple instrumentation requirements. Solid-phase immunoassays are more widely used. Such systems are called enzyme-linked immunosorbent assays (ELISAs). The ELISA can be used to detect and determine concentrations of antigen or antibody. The test may be done in polystyrene well (microtitre plate) or tube. Different types of ELISA have been developed (Fig. 13.11).

1. Indirect ELISA

The principle of this test can be illustrated by outlining its application for detection of anti-HIV-1 and anti-HIV-2 antibodies in the patient serum. The wells of the polystyrene microtitre plate are coated with purified HIV-1 and HIV-2 antigens or synthetic peptides representing immunodominant epitopes of HIV-1 and HIV-2, which constitutes the solid-phase antigen. Diluted test serum or plasma sample is added to such a well and incubated. If antibodies specific for HIV-1 and/or HIV-2 are present in the test sample they will form stable complexes with antigens coated on the well. Well is then washed and a conjugate of goat antihuman immunoglobulin, which has been labelled with the enzyme horse-

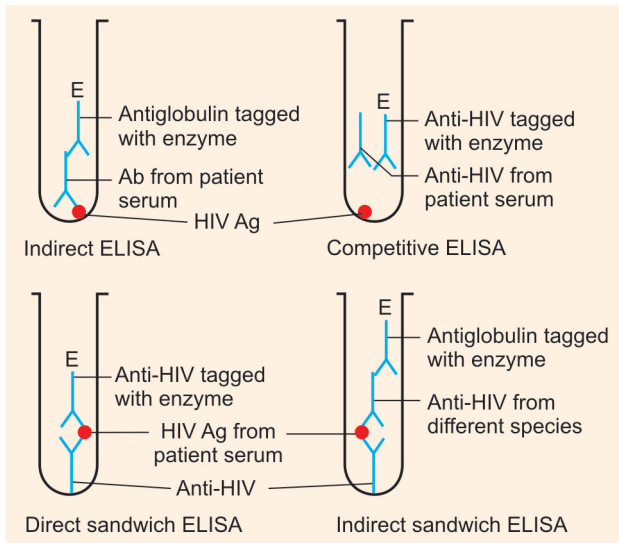


Fig. 13.11: ELISA.

radish peroxidase, is added. If the antigen–antibody complex is present, the peroxidase conjugate will bind to the complex and remains in the well. The conjugate fraction remaining free in the well is removed by washing and the presence of enzyme immobilized on the complexes is shown by incubation in the presence of a colourless enzyme substrate (ortho phenylenediamine dihydrochloride solution). Incubation with enzyme substrate produces a yellow–orange colour in the test well. If the sample contains no anti-HIV-1 and/or anti-HIV-2, then the labelled antibody cannot be found and no colour develops. The absorbance value of each well is read by an ELISA plate reader at wavelength of 492 ± 2 nm.

2. Competitive ELISA

The principle of this test too can be illustrated by outlining its application for detection of anti-HIV antibodies in the patient serum. The wells of the polystyrene microtitre plate are coated with HIV antigens which constitutes the solid-phase antigen. The test sample and human anti-HIV, which has been labelled with the enzyme horseradish peroxidase, are incubated in such a well. When the sample contains no anti-HIV, solid-phase antigen-labelled antibody complex will be formed. The incubation with enzyme substrate produces a yellow–orange colour in the test well. If anti-HIV is present in the test sample, it competes with the labelled antibody for the available solid-phase antigen and no colour or reduced colour develops. Competitive ELISA takes less time than indirect ELISA and no predilution of test serum is required.

3. Sandwich ELISA

The most frequently used ELISA for detecting microbial antigen is the sandwich solid-phase ELISA. It is of two types:

(i) Single antibody or direct sandwich ELISA

Antibody is attached to the solid-phase. The test sample is then exposed to the solid-phase antibody, to which the antigen, if present, will bind. The solid-phase antibody–antigen complex is then rinsed free of unbound test sample

and exposed again to antibodies reactive against the test antigen and conjugated with the enzyme. The conjugated antibody will react with the antigen held to the solid-phase by the first antibody, forming an antibody–antigen–antibody sandwich on the solid-phase. The solid-phase sandwich is again separated from unreacted test sample by rinsing. The second antibody (conjugated to an enzyme) can be detected with an appropriate substrate. This is a single antibody or direct sandwich ELISA.

(ii) Double antibody or indirect sandwich ELISA

In the double antibody ELISA the second antibody as above is not conjugated with the enzyme. The second antibody can be detected by treating it with an anti-immunoglobulin–enzyme conjugate. In the double antibody ELISA, the second antibody of the sandwich must be from a different species than the solid-phase antibody, otherwise, the anti-immunoglobulin conjugate reacts with the solid-phase antibody, producing high background activity.

ELISA is a simple and versatile technique. It needs only microlitre quantities of reactants. ELISA kits are commercially available for the detection of anti-HIV, hepatitis B surface antigen and rotavirus. ELISA kits have also been developed for detecting:

- *Entamoeba histolytica* antigens in faeces.
- *Toxoplasma* antigens in the patient serum.
- *Haemophilus influenzae* antigens in spinal fluid.
- β -haemolytic streptococcal antigens in spinal fluid.
- Hepatitis A virus in stools.
- Respiratory syncytial virus in pharyngeal secretions.
- Adenovirus antigens in nasopharyngeal specimens.
- Labile enterotoxin of *Escherichia coli* in stools.

Chemiluminescence immunoassay (CLIA)

Chemiluminescence refers to a chemical reaction emitting energy in the form of light. As radioactive conjugates are employed in RIA, fluorescent conjugates in fluorescence microscopy and enzymes in ELISA, chemiluminescent compounds such as luminol or acridinium esters are used in CLIA as the label to provide the signal during the antigen–antibody reaction. The signal (light) can be amplified, measured and the concentration of analyte calculated. The method has been fully automated.

Western blotting

Western blotting is analogous to Southern blotting, for isolated DNA, and Northern blotting, for isolated RNA. In Western blotting, protein antigens are separated according to their electrophoretic mobility and molecular weight by polyacrylamide gel electrophoresis, then blotted onto nitrocellulose paper by standard blotting procedure. The patient serum is allowed to react with the blot. Antibodies attached to separated viral antigens on the nitrocellulose paper are detected by enzyme tagged-antihuman gamma-globulin. Enzyme substrate is subsequently added which indicates positive test. The substrate changes colour in the

presence of enzyme and permanently stains the nitrocellulose paper. The position of the band on the paper indicates the antigen with which the antibody has reacted.

Immunoelectron microscopy

When the virus particles, for example, rotavirus and hepatitis A virus in stool, are scanty in the specimen they can be treated with specific antisera. It leads to clumping of virus particles which can be seen under electron microscope. This is known as immunoelectron microscopy and it finds application in detection of some viruses causing diarrhoea.

Capsule swelling or Quellung reaction

Mixing capsulated bacteria, such as *S. pneumoniae* and *Klebsiella pneumoniae*, with homologous antibody makes

possible the direct microscopic visualization of capsules. Binding of the homologous type specific antibody increases the refractility and apparent thickness of the capsule thus making direct microscopic visualization of the capsule possible.

Immunoenzyme test

Some stable enzymes, such as peroxidases, can be conjugated with antibodies. Antigens in tissue sections can be detected by treating them with peroxidase conjugated specific antibody. If the tissue section possesses specific antigen then antigen-enzyme conjugated antibody complexes will be formed which can be detected by treatment with enzyme substrate.

KEY POINTS

- **Antigen–antibody reactions are specific** but cross-reactions may occur.
- When a **soluble antigen** is mixed with its **specific antibody** in the presence of **electrolytes** at a **suitable temperature** and **pH**, the antigen–antibody complex forms an **insoluble precipitate**.
- When a **particulate antigen** or an **antigen** present on a cell or an inorganic particle is mixed with its **antibody** in the presence of **electrolytes** at a **suitable temperature** and **pH**, the particles are **clumped** or **agglutinated**.
- The ability of **antigen–antibody complexes** to **fix complement** is made use of in **complement fixation test**.
- A substance, such as complement or antibody, that can bind to the surface of a cell or a particle, making it more readily phagocytosed is known as **opsonin**.
- Radioactive conjugates are employed in radioimmunoassay, fluorescent conjugates in fluorescence microscopy, enzymes in enzyme-linked immunosorbent assay and chemiluminescent compounds are used in chemiluminescence immunoassay.



Important Questions

1. Define antigen–antibody reaction. Name various antigen–antibody reactions and describe the principle and applications of precipitation reaction giving suitable examples.
2. Define agglutination reaction and discuss the principle and applications of agglutination reactions giving suitable examples.
3. Describe the principle of complement fixation test and discuss in brief the various complement dependent serological tests.
4. Discuss the principle and clinical applications of immunofluorescence technique.
5. Discuss the principle, various types and clinical applications of ELISA technique.
6. Discuss the principle and clinical applications of Western blot technique.
7. Define the terms affinity, avidity, specificity and sensitivity of a test.
8. Discuss prozone, zone and postzone phenomenon.
9. Write short notes on:

(a) Neutralization	(b) Opsonization
(c) Radioimmunoassay	(d) Chemiluminescence assay



Multiple Choice Questions

1. Ring test is used for:

(a) typing of streptococci and pneumococci.	2. VDRL test is an example of:
(b) C-reactive protein test.	(a) ring test.
(c) Ascoli's thermoprecipitation test.	(b) slide flocculation test.
(d) all of the above.	(c) tube test.
	(d) none of the above.

3. Agglutination reaction is more sensitive than precipitation for detection of:
 - (a) antigen.
 - (b) antibody.
 - (c) antigen–antibody complex.
 - (d) complement.
4. Incomplete antibodies may be detected by doing the test:
 - (a) in hypertonic saline.
 - (b) in albumin saline.
 - (c) by the antiglobulin test.
 - (d) all of the above.
5. Tube agglutination is employed for serological diagnosis of:
 - (a) enteric fever.
 - (b) brucellosis.
 - (c) typhus fever.
 - (d) all of the above.
6. Anti-Rh antibodies are:
 - (a) IgG type.
 - (b) IgD type.
 - (c) IgA type.
 - (d) IgE type.
7. A precipitation reaction can be converted into agglutination reaction by coating soluble antigen onto:
 - (a) RBCs.
 - (b) latex particles.
 - (c) bentonite particles.
 - (d) all of the above.
8. Test employing complement is:
 - (a) Widal test.
 - (b) VDRL test.
 - (c) *Treponema pallidum* immobilization test.
 - (d) ELISA.
9. Quelling reaction is used for typing of:
 - (a) *Streptococcus pneumoniae*.
 - (b) *Klebsiella pneumoniae*.
 - (c) both of the above.
 - (d) none of the above.
10. Widal test is an example of:
 - (a) agglutination test.
 - (b) precipitation test.
 - (c) flocculation test.
 - (d) complement-fixation test.
11. An agglutination reaction which makes use of *Staphylococcus aureus* bound IgG as a reagent is known as:
 - (a) passive agglutination.
 - (b) reversed passive agglutination.
 - (c) coagglutination.
 - (d) flocculation.
12. Coombs' test is:
 - (a) complement fixation test.
 - (b) antiglobulin test.
 - (c) agglutination test.
 - (d) precipitation test.
13. Inactivation of a toxin by an antibody is known as:
 - (a) opsonization.
 - (b) neutralization.
 - (c) attenuation.
 - (d) lyophilization.



Answers

1. d 2. b 3. b 4. d 5. d 6. a 7. d 8. c 9. c 10. a 11. c 12. b
13. b

Architecture of the Immune System

Competency achievement: The student after reading the chapter should be able to:

PA9.1: Describe the principles and mechanisms involved in immunity.

Immune responses are mediated by a variety of cells, and by the soluble molecules which they secrete. Lymphocytes (B cells and T cells), phagocytes (mononuclear phagocytes, neutrophils and eosinophils), and auxiliary cells (basophils, mast cells and platelets) are the cellular components of immune system. *Antibodies produced by B cells, cytokines produced by T cells and mononuclear phagocytes, complement produced by mononuclear cells, inflammatory mediators produced by basophils, mast cells and platelets, and interferons produced by infected tissue cells are the soluble mediators of immune system.*

Immune response to an antigen is of two types:

- **Humoral or antibody-mediated immunity (AMI)** which is mediated by antibodies produced by plasma cells.
- **Cell-mediated immunity (CMI)** which is mediated directly by sensitized lymphocytes.

Lymphoid organs can be classified into primary (central) lymphoid organs and secondary (peripheral) lymphoid organs. Thymus and bursa of Fabricius are primary lymphoid organs. They are responsible for cellular and humoral immune response, respectively. The equivalent of the avian bursa of Fabricius, in mammals, is bone marrow.

The capacity to respond to immunologic stimuli resides mainly in lymphoid cells. During embryonic development, blood cell precursors are found in foetal liver and other tissues; in postnatal life, the stem cells reside in bone marrow. They can differentiate in several ways. In liver and bone marrow, stem cells may differentiate into cells of red cell series or into cells of lymphoid series. Lymphoid stem cells evolve into two main lymphocyte population, B cells and T cells. If a stem cell is to become a T cell, it leaves the bone marrow and emigrates to the thymus where it differentiates further under the influence of the thymic microenvironment and soluble factors produced by the thymic epithelium. The resulting T cells are responsible for specific cell-mediated immunity.

However, if the lymphoid stem cell is destined to become a B cell it remains in the bone marrow (in case of birds it emigrates to bursa of Fabricius, a gut appendage) where it undergoes several more differentiative steps before it gains the ability to produce and secrete antibody in response to the presence of infectious organisms. After acquiring immunocompetence, both T and B cells leave their primary site of differentiation and emigrate to the peripheral lymphoid organs (Fig. 14.1). These include lymph nodes, spleen, gut-associated lymphoid tissue (GALT), appendix, tonsils and adenoids.

B cells seed into outer cortex in germinal follicles and medullary cords of peripheral lymph nodes and germinal centre and mantle layer of spleen. These areas are known as **bursa-dependent** or **thymus-independent areas**. T cells seed into paracortical areas of lymph nodes and white pulp of spleen around the central arterioles. These areas are known as **thymus-dependent areas**. Here, in the peripheral lymphoid organs, they encounter with infectious organisms that have escaped the innate defence system.

PRIMARY LYMPHOID ORGANS

Thymus

It is a bilobed, greyish, lymphoepithelial organ located just above the heart and extending into the neck on the front and sides of trachea. It develops from the epithelium of third and fourth pharyngeal pouches at about the sixth week of gestation. The embryonic thymus consists of epithelial cells surrounded by a thin capsule. *In utero*, precursor cells (prothymocytes or progenitor lymphocytes) differentiate from the lymphoid stem cell, emigrate from bone marrow and infiltrate the thymus, wedging between the epithelial cells to form a meshwork of branched epithelial cells and lymphocytes. The peripheral epithelium, called the cortex, becomes heavily populated with the resulting thymocytes, whereas the central area, termed the medulla contains few lymphocytes some of which are derived from the cortical thymocytes.

Histologically, each lobe of thymus is divided into a series of lobules each containing a cortex and a medulla. Prothymocytes, from the bone marrow, migrate through bloodstream, enter the cortex and begin dividing rapidly. It is the major site for lymphocyte proliferation in the body. However, most

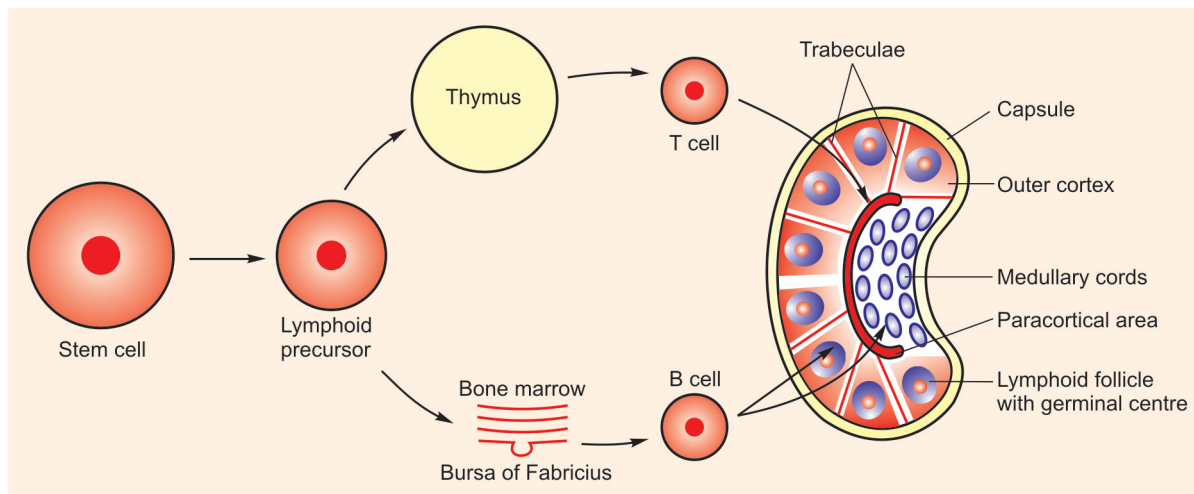


Fig. 14.1: Development of T and B cells.

cells die in the process. Of the lymphocytes produced only 5% leave the thymus as viable cells. The reason for this apparent wasteful process is not known. However, some believe that this apparent waste may represent the elimination of lymphocyte clones that react against self. In the thymus, the lymphocytes acquire new surface antigens (*Thy antigens*). Lymphocytes produced in the thymus are called **thymus-derived lymphocytes** or **T lymphocytes** or **T cells**. Unlike lymphocytes proliferation in the peripheral organs, in the thymus, it is not dependent on antigenic stimulation.

The thymus confers immunological competence on the lymphocytes during their stay in the organ. Here they are educated, so that they become capable of mounting cell-mediated immune response against appropriate antigen. This is effected under the influence of the thymic microenvironment and several hormones, such as thymosin and thymopoietin produced by thymic epithelium. Differentiation of thymocytes to T cells, in the thymus, is immediately followed by emigration of these T cells to the peripheral lymphoid organs as mature cells that are precommitted to their function and antigen specificity. These are selectively seeded into paracortical areas of peripheral lymph nodes and into the white pulp of the spleen around the central arterioles. These regions are known as '**thymus-dependent**' and after neonatal thymectomy it is found grossly depleted.

The thymus reaches peak activity in childhood and attains its largest size at puberty. Thereafter, the thymus begins to atrophy without any apparent effect on T cell function and is extremely small in old age. This is probably due to the fact that T cells are very long-lived and can circulate in the resting state for long periods of time.

Bursa of Fabricius

The bursa is a lymphoepithelial organ arising as a pouch from the dorsal part of the cloaca in birds. Like thymus, it is also a site of lymphocytic proliferation and differentiation. Stem cells from yolk sac, foetal liver and bone marrow enter the bursa, proliferate and develop into immunocompetent **bursal lymphocytes** or **B lymphocytes** or **B cells** (B for

bursa or bone marrow). These B cells now migrate and seed outer or superficial cortex of the germinal follicles and medullary cords of peripheral lymph nodes and lymphoid follicles of spleen. These are known as '**bursa-dependent**' or **thymus-independent areas**. Following appropriate antigenic stimulation, B lymphocytes transform into plasma cells and secrete antibodies. Surgical removal of bursa (bursectomy) from newly hatched chickens destroys their subsequent ability to produce antibodies but does not affect their ability to mount cell-mediated immune response. Like thymus, the bursa starts to shrink or atrophy at puberty.

Bone marrow

The mammalian equivalent of the bursa of Fabricius appears to be the bone marrow. Therefore, mammalian bone marrow is the site not only of haemopoiesis but also of initial differentiation of stem cells to B cells.

PERIPHERAL LYMPHOID ORGANS

Lymphocytes differentiate and mature in the primary lymphoid organs and proceed via circulation to the secondary lymphoid organs. Here they have an opportunity to bind antigen and undergo further antigen-dependent differentiation. Once in the secondary lymphoid tissues, the lymphocytes do not remain there but move from one lymphoid organ to another through the blood and lymphatics. The advantage of this lymphocyte recirculation is that during the course of a natural infection the continual trafficking of lymphocytes enables many different lymphocytes to have access to antigen. The passage of lymphocytes through an area where antigen has been localized and concentrated on the dendritic processes of macrophages or on the surface of antigen-presenting cells facilitates the induction of an immune response.

Lymph nodes

Lymph nodes are small bean-shaped organs that act as filters. They form part of lymphatic network distributed throughout the body. They are surrounded by connective tissue capsules

from which trabeculae penetrate into the nodes. They consist of an outer cortex and an inner medulla. The cortex consists of several rounded aggregates of lymphocytes called lymphoid follicles. The follicle has a pale-staining germinal centre surrounded by small dark-staining lymphocytes. The follicles contain besides proliferating lymphocytes, dendritic macrophages, which capture and process the antigen.

The deeper region of the cortex or paracortex is the zone between the peripheral cortex and inner medulla. The medulla is predominantly composed of cords (medullary cords) of lymphocytes. Lymphoid follicles and medullary cords contain B lymphocytes and constitute **bursa-dependent areas** while paracortex (paracortical area) contains T lymphocytes and constitute **bursa-independent areas**. Each lymph node has a number of lymph channels called afferent lymph channels, that drain into it and a single large lymph vessel, called efferent lymph channel that carries the lymph fluid and lymphocytes to the thoracic duct, which empties into a large vein in the neck.

Spleen

Spleen is a large, encapsulated, lymphoid organ within which antibody synthesis to most blood-borne antigens takes place. There are two types of tissues in the spleen, referred to as the lymphoid or white pulp of the cortex consisting primarily of lymphocytes and macrophages, and the erythroid or red pulp, of the medulla consisting of erythrocyte-rich blood. Arteries entering the spleen are surrounded by a sheath of T cells and macrophages. This is known as periarteriolar sheath. Within this sheath are present lymphoid follicles known as primary follicles of B cells similar to those occurring in lymph nodes. Blood-borne antigens entering the spleen are phagocytosed and processed by macrophages and fixed phagocytic mononuclear cells. Presentation of antigens, on the surface of such cells, to the splenic lymphocytes results in the formation of secondary follicles containing germinal centres of dividing and differentiating B cells.

Mucosa-associated lymphoid tissues

Mucosa lining the alimentary, respiratory, genitourinary and other surfaces are constantly exposed to numerous antigens. These areas possess rich collection of lymphoid tissues. These collections of lymphoid tissues are known as mucosa-associated lymphoid tissue (MALT). Tonsils, adenoids and Peyer's patches of the small intestine are known as gut-associated lymphoid tissue (GALT). Peyer's patches are small patches of organized lymphoid tissue along the intestine containing B cells (in germinal centre) and T cells. They play a primary role in defence against infectious organisms entering via digestive tract.

CELLS OF THE IMMUNE SYSTEM

Lymphocytes

Of the many cells involved in specific response to antigen, lymphocytes are the most important effector cells. They are small, round, 5–15 µm in diameter and are found in peripheral blood, lymph, lymphoid organs and in many other tissues.

In peripheral blood, they constitute 20–40% of the leucocyte population, while in lymph and lymphoid organs they form the predominant cell type. They may be small (5–8 µm), medium (8–12 µm) and large (12–15 µm). The small lymphocytes are most numerous. They may be short-lived (lifespan about 2 weeks) or long-lived (lifespan 3 years or more or even for life). Short-lived cells are effector cells in immune response, while long-lived cells act as memory cells. Long-lived cells are mainly thymus-derived. Lymphopoiesis takes place in the bone marrow, central lymphoid organs and peripheral lymphoid organs.

Lymphocytes possess antigen recognition mechanism on their surface, enabling each cell to recognize only one or a small number of antigens. Two major classes of lymphocytes are recognized which are designated **T cells** and **B cells**. T and B cells are indistinguishable by conventional light microscopy.

Classification of lymphocytes on the basis of surface markers makes use of two important characteristics:

1. Cluster of differentiation or cluster determinant (CD).
2. Antigen recognition receptors.

1. Cluster of differentiation or cluster determinant

CDs represent families of surface glycoprotein antigens that can be recognized by specific antibodies produced against them. Thus, a cell displaying CD1 is identified by the binding of antibodies against CD1. Each class of leucocyte displays a diagnostic pattern of CDs, for example:

- CD3 is expressed only by T cells.
- CD19 is expressed only by B cells.
- CD64 is expressed only by monocytes.
- CD66 is expressed only by granulocytes.
- CD68 is expressed only by macrophages.
- On the other hand, CD18 and CD45 are expressed by a variety of leucocyte types.
- A total of more than 150 CDs are known.

2. Antigen recognition receptors

These include membrane-bound (surface) immunoglobulins (mIgs or sIgs) in B cells, and T cell receptors (TCRs) in T cells. In contrast to CDs, which can serve as diagnostic feature for all leucocytes, antigen recognition receptors are limited to B and T lymphocytes only. These receptors are required for B and T cells to be antigen reactive. Both mIgs and TCRs serve as specific surface receptors, recognizing and interacting with only single antigenic determinant on the antigen. Reaction of antigens with mIgs and TCRs activates B cells and T cells, respectively, leading to proliferation and differentiation.

Thus, the antigen specificity of the mIgs in B cells and TCRs in T cells is predetermined, and the sole effect of antigen is to select out a cell with appropriate surface receptor and induce it to clonally expand and differentiate into a cell that will produce the antibody it has been predetermined to make or produce specific clones of effector T cells, respectively.

B lymphocytes

Lymphocytes possessing mIgs are termed B cells. They arise from pluripotent stem cells in bone marrow, they mature in bone marrow itself and then emigrate to the peripheral lymphoid organs where, upon contact with antigen, they can differentiate into antibody-producing **plasma cells**. Antibodies are formed by clonal selection. Each individual has a large pool of different B lymphocytes (about 10^9) that have life-span of days or weeks and are formed in the bone marrow, lymph nodes, and gut-associated lymphoid tissue (e.g. tonsils or appendix). Each B cell possesses about 10^5 mIg molecules, primarily of IgM and IgD classes. Following activation, immunoglobulins of other classes might also reside on B lymphocyte membranes. Immature B cells do not possess mIg receptors.

A single B cell or a clone of B cells possess mIg receptors specific for only one (monospecific) antigenic determinant. Thus, billions of B cells display a diversity of receptors capable of reacting with any antigenic determinant that might be encountered. Receptor immunoglobulin and secreted immunoglobulin of a single cell or clone of cells are identical in the variable regions of the antibody molecule.

Most B cells and macrophages, and certain activated T cells express class II major histocompatibility gene products or immune associated (Ia) antigens in the mouse, and HLA-DR antigens in humans. Receptors for the Fc portion of IgG (FcR) are found on all B cells, macrophages and certain subsets of T cells. Some cells express receptors for other classes of immunoglobulin as well. These receptors bind antigen-antibody complexes. Receptors for C3 component of complement are found on most B cells. These are known as complement receptors (CRs). These receptors are thought to play a role in the regulation of the B cell response to antigen. Because of the presence of CRs on the surface of B cells they bind to sheep RBCs which have been coated with antibody and complement forming EAC (erythrocyte antibody complement) rosettes. They undergo blast transformation on treatment with bacterial endotoxins.

T lymphocytes

Pluripotent stem cells in the bone marrow give rise to precursor T cells, which migrate to the thymus (Fig. 14.2). Once they enter the cortex of the thymus they are known as **thymocytes**. As T cells mature, their surface antigens including CDs and TCRs change. Monoclonal antibodies are used to identify the antigenic subsets of T cells. Approximately 65% of mature T cells that leave the thymus display CD2⁺ CD3⁺ CD4⁺ CD8[−] TCR⁺ phenotype (CD4⁺ cells), while approximately 35% display CD2⁺ CD3⁺ CD4[−] CD8⁺ TCR⁺ phenotype (CD8⁺ cells). A very small number express neither CD4 nor CD8 and consequently have a phenotype of CD2⁺ CD3⁺ CD4[−] CD8[−] TCR⁺ (CD4[−] CD8[−] cells).

T cell subsets

Four distinct subsets of T cells are known. Two each of these are regulator and effector cells.

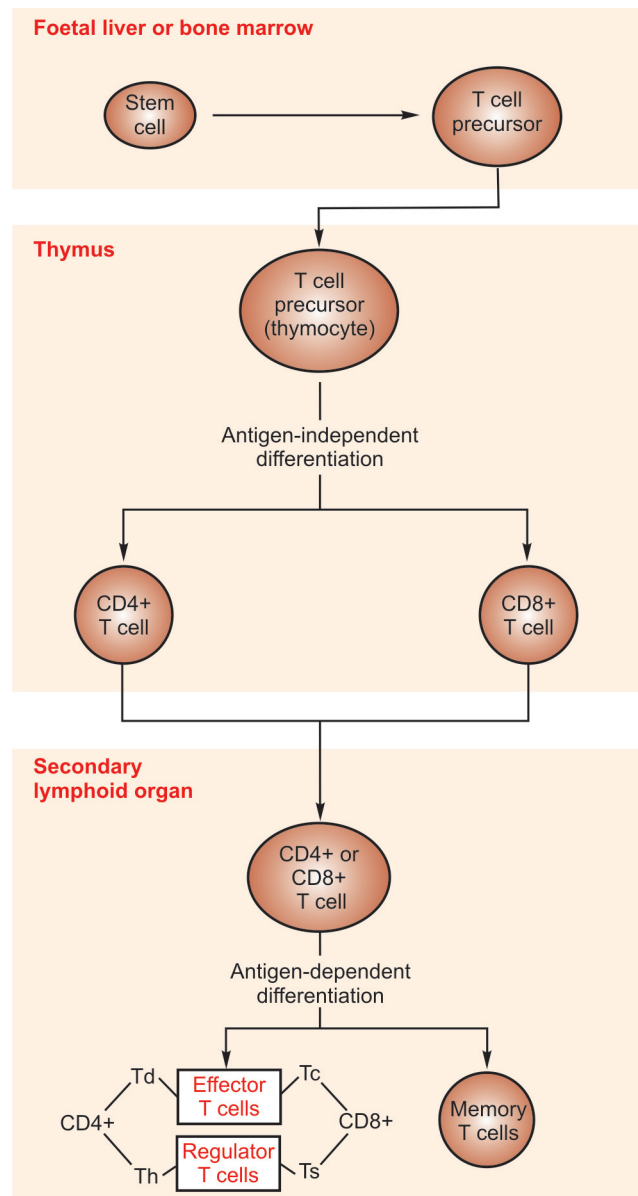


Fig. 14.2: T cell differentiation.

Regulator cells

1. **Helper T cells (Th cells):** They possess CD2, CD3 and CD4 surface antigens. They help in the antigen-specific activation of B cells and effector T cells. Th cells have two different profiles of cytokine production (Th1 and Th2) and these patterns select between the two basic types of response mediated by Th cells. Th1 cytokines include interferon- γ (IFN- γ), IFN- β , and interleukin-2 (IL-2), and Th1 cells are involved in cell-mediated inflammatory reactions. Several Th1 cytokines activate cytotoxic inflammatory and delayed hypersensitivity reactions. By contrast, Th2 cells are typified by the production of IL-4 to 6, IL-9, IL-10 and IL-13. Th2 cells encourage production of antibody especially IgE, and Th2 cytokines are associated with regulation of strong antibody and allergic responses. Cytokines from Th1 cells inhibit the actions of Th2 cells and vice versa.

2. *Suppressor T cells (Ts cells)*: They possess CD2, CD3 and CD8 surface antigens. They suppress expression of immune response by other lymphocytes.

Effector cells

1. *Delayed-type-hypersensitivity T cells (Td cells)*: They possess CD2, CD3 and CD4 surface antigens. They are involved in delayed hypersensitivity and cell-mediated immune responses.
2. *Cytotoxic T cells (Tc cells)*: They possess CD2, CD3 and CD8 surface antigens. They are also involved in cell-mediated immune responses and lyse target cells by direct cell-cell contact.

Immune response is regulated by mutually opposing influence of Th and Ts cells. **Overactivity of Th or decreased activity of Ts causes abnormal immune responses as seen in autoimmunity. Diminished Th function or increased activity of Ts leads to immunodeficiency.**

During maturation and differentiation in thymus, T cells also learn to recognize self-major histocompatibility (MHC) antigens. CD4⁺ cells recognize class II MHC antigens and CD8⁺ cells recognize class I MHC antigens.

T cells bind to sheep RBCs at 37°C forming SRBC or E rosettes while B cells do not. They undergo blast transformation, evidenced by enhanced DNA synthesis, on treatment with mitogens such as phytohaemagglutinin (PHA) and concanavalin A (Con A).

Table 14.1 summarises differences between T cells and B cells.

Table 14.1: Differences between T and B cells

Property	T cell	B cell
Antigen recognition receptors	TCRs	mIgs
Surface glycoprotein antigens	CD3	CD19
Receptors for Fc piece of immunoglobulins (FcR)	—*	+
Receptors for C3 component of complement (CRs)	—	+
EAC rosette	—	+
E rosette	+	—
Thymus specific antigens	+	—
Blast transformation on treatment with	PHA and Con A	Bacterial endotoxins

* Certain subsets of T cells possess FcR

Null cells

A small proportion (5%) of lymphocytes that lack distinguishing phenotypic markers characteristic of T or B lymphocytes are known as null cells or non-T and non-B lymphocytes. They do not possess TCRs or mIgs. A few null cells in the circulation might be immature T or B cells.

Killer cells or K cells

A subpopulation of null cells possess surface receptors for Fc part of IgG. They are capable of lysing or killing target cells sensitized by IgG antibody. They are known as killer or

K cells. **They are responsible for antibody-dependent cell-mediated cytotoxicity (ADCC) in contrast to the action of cytotoxic T lymphocytes which are independent of antibody.**

Natural killer or NK cells

Another subpopulation of null cells is natural killer or NK cells. These are large lymphocytes containing azurophilic granules in the cytoplasm. They are, therefore, known as large granular lymphocytes (LGL). **NK cells are capable of non-specific killing of virus-transformed target cells and are involved in allograft and tumour rejection.** They differ from K cells in being independent of antibody.

Plasma cells

Plasma cells are fully differentiated antibody-synthesizing cells. Antigenically stimulated B cells undergo blast transformation, becoming successively plasmablasts, intermediate transitional cells and plasma cells. It is an oval cell, about twice the size of a small lymphocyte. It has an eccentric nucleus, abundant rough endoplasmic reticulum, numerous mitochondria and prominent Golgi apparatus. Plasma cells are end cells and have a short life span of 2 or 3 days. A plasma cell secretes an antibody of a single specificity of a single antibody class and of a single light chain type. However, in primary antibody response plasma cell produces IgM initially and later it may switch onto IgG production. Lymphocytes, lymphoblasts and transitional cells may also synthesize immunoglobulins to some extent.

Antigen-presenting cells (APCs)

A number of different cell types have been described as APCs. In addition to presenting antigen to effector lymphocytes, many of these cells perform non-specific immunological functions such as phagocytosis and cytotoxicity. Induction of humoral or cell-mediated immunity cannot occur efficiently in the absence of APCs, i.e. with lymphocytes alone. APCs include dendritic cells that are found in skin (Langerhans' cells), thymus, lymph node, spleen and other secondary lymphoid organs and macrophages which include monocytes as blood macrophages and histiocytes as tissue macrophages.

The processing and presentation of antigen by macrophages to T cells require that both the cells possess surface determinants coded by the same MHC genes. T cells can accept the processed antigen only if it is presented by macrophages carrying on its surface the self-MHC determinant known as immune-associated or Ia antigen. When the macrophage bears a different Ia antigen, it cannot cooperate with T cell. This is known as **MHC restriction**.

Functional activity of macrophages may be enhanced by lymphokines, complement components and interferon. Activated macrophages are not antigen specific. They secrete a number of biologically active substances like interleukin-1. They can bind immune complexes by means of FcRs or CRs, which are present on their surface, and then engulf and digest them. Macrophages can also exhibit ADCC reactions. Role of macrophages in innate immune response is discussed in Chapter 9.

Other cells involved in immunological responses

Neutrophils

Approximately 60% of the circulating leucocytes in humans are neutrophils. Their primary function is phagocytosis of foreign or dead cells and pinocytosis of pathological immune complexes. They can also exhibit ADCC. They are capable of rapid activation and mobilization in response to chemotactic stimuli such as bacterial products or activated components of complement (C5a). A variety of receptors, e.g. FcRs, CR1 and CR2 are increasingly displayed following activation. They constitute predominant cell type in inflammation.

Eosinophils

These are granulocytes containing prominent acidophilic granules. They account for 3–5% of the white blood cells.

During allergic conditions and during certain parasitic infections the number of eosinophils may increase dramatically. They can engulf and remove immune complexes by phagocytosis/pinocytosis. They possess FcRs and can mediate ADCC. They can bind to worm larvae such as schistosomulae coated with IgG, degranulate and release toxic proteins which are damaging to the parasites.

Basophils and mast cells

Basophils comprise less than 1% of white blood cells. Basophils and their tissue counterparts, mast cells, possess basophilic granules. These granules contain pharmacological mediators of type I hypersensitivity. IgE antibodies get attached to FcRs present on the surface of mast cells and basophils. When stimulated with allergen, granules release their contents. Basophils also possess FcRs for IgG and CRs for C3a, C3b and C5a.

KEY POINTS

- **The immune system** is able to recognize and respond to many antigens by generating great diversity in the antibodies produced by B cells.
- Each lymphocyte expresses either antibody or a TCR with a single specificity for antigen.
- A lymphocyte bearing a complementary antibody or TCR on its surface will bind antigen, be activated, proliferate to form a clone, and also form a large pool of memory cells.
- Many antigens require T cell help before they can activate B cells, and the interactions are mediated by a variety of soluble cytokines.
- **Immune response** to antigen is of two types—humoral or antibody mediated immunity and cell-mediated immunity.
- **T cell subsets** include regulator cells (Th cells and Ts cells) and effector cells (Td cells and Tc cells).



Important Questions

1. Name primary and secondary lymphoid organs and discuss the role of thymus in immune response.
2. Name various cells of the immune system. Describe briefly classification systems used to differentiate lymphocytes.
3. Differentiate between T and B cells in a tabulated form.
4. Write short notes on:
 - (a) Subsets of T lymphocytes
 - (b) Null cells
 - (c) Plasma cells
 - (d) Bursa of Fabricius
 - (e) Mucosa-associated lymphoid tissue



Multiple Choice Questions

1. Primary lymphoid organ/s is/are:
 - (a) thymus.
 - (b) bursa of Fabricius.
 - (c) bone marrow.
 - (d) all of the above.
2. Secondary lymphoid organ/s is/are:
 - (a) lymph nodes.
 - (b) spleen.
 - (c) mucosa-associated lymphoid tissue.
 - (d) all of the above.
3. Stem cells arise from:
 - (a) yolk sac.
 - (b) foetal liver.
 - (c) bone marrow.
 - (d) all of the above.
4. Lymphoid follicles of lymph nodes are:
 - (a) bursa-dependent areas.
 - (b) bursa-independent areas.
 - (c) thymus-dependent areas.
 - (d) none of the above.

5. CD3 surface antigen is expressed only by:
(a) T cells.
(b) B cells.
(c) monocytes.
(d) granulocytes.
6. CD8 surface antigen is present in:
(a) suppressor T cells.
(b) cytotoxic T cells.
(c) both of the above.
(d) none of the above.
7. EAC rosettes are formed by:
(a) T cells.
(b) B cells.
(c) monocytes.
(d) granulocytes.
8. Blast transformation on treatment with bacterial endotoxins occurs in:
(a) T cells.
(b) B cells.
(c) monocytes.
(d) none of the above.
9. Natural killer cells are involved in:
(a) allograft rejection.
(b) tumour rejection.
(c) non-specific killing of virus transformed target cells.
(d) all of the above.
10. Which of the following immune cells/molecules are most effective in destroying intracellular pathogens?
(a) Th cells.
(b) Tc cells.
(c) B cells.
(d) antibodies.
11. B cells that produce and release large amounts of antibody are called:
(a) memory cells.
(b) killer cells.
(c) basophils.
(d) plasma cells.



Answers

1. d 2. d 3. d 4. a 5. a 6. c 7. b 8. b 9. d 10. b 11. c

Immune Response

PA9.1: Describe the principles and mechanisms involved in immunity.

Specific reactivity following an antigenic stimulus is known as the immune response. It is of two types:

1. Humoral or antibody-mediated immunity (AMI).
2. Cell-mediated immunity (CMI).

AMI provides defence against most extracellular bacterial pathogens and helps in defence against viruses that infect through the respiratory and intestinal tract. It also participates in immediate (types I, II, III and V) hypersensitivity reactions and certain autoimmune diseases.

CMI protects against fungi, most of the viruses and intracellular bacterial pathogens like *Mycobacterium leprae*, *M. tuberculosis*, *Brucella* and *Salmonella*, and parasites like *Leishmania* and trypanosomes. It also participates in allograft rejection, graft versus host reaction, delayed hypersensitivity and certain autoimmune diseases. It provides immunological surveillance and immunity against cancer.

HUMORAL OR ANTIBODY-MEDIATED IMMUNE RESPONSE

The antibody response to stimulation by antigen can be described as primary humoral response and secondary humoral response.

Primary humoral response

Phases

Antibody production follows characteristic phases (Fig. 15.1):

1. **Lag phase:** After first injection of the antigen there is a long lag phase of several days before antibody appears. The lag phase depends upon the kind and amount of antigen given, the route of administration, species of animal and its health.
2. **Log phase:** As the lag period ends, the titre of antibody gradually increases over a period of a few days to a few weeks.
3. **Plateau or steady state:** There is equilibrium between antibody production and catabolism.

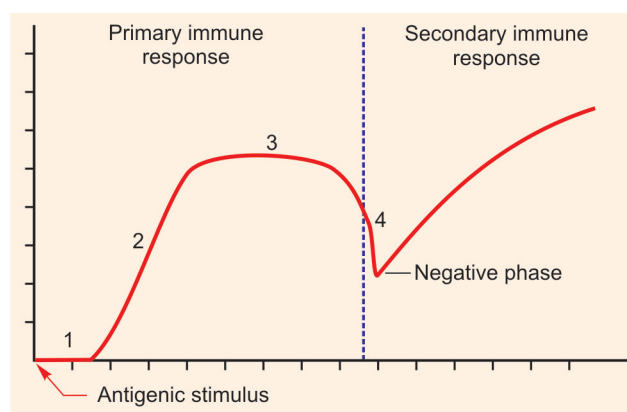


Fig. 15.1: Immune response.

4. **Phase of decline:** Catabolism exceeds the production of antibody and the titre falls.

Secondary humoral response

If the same animal is subsequently exposed to the same antigen there occurs a temporary fall in the level of antibodies due to the combination of the antigen with pre-existing antibody. This is known as **negative phase**. After 2 to 3 days a marked increase in antibody level becomes evident. This goes on increasing for several days, thus exceeding the initial level. This is also known as **booster response**. The booster response is attributed to the persistence of antigen sensitive 'memory cells' following the primary response.

The antibody formed in primary response is predominantly IgM and in secondary response IgG. The first dose is known as **priming dose** and subsequent injection as **booster dose**. Both these doses are particularly essential in case of killed vaccines. *With live vaccines a single dose is usually sufficient as multiplication of organisms in the body provides a continuing antigenic stimulus that acts both as priming and booster doses.*

Fate of antigen in tissues

Antigens introduced subcutaneously are mainly localized in the draining lymph nodes, only a small amount being found

in the spleen. On the other hand, antigens introduced intravenously are rapidly localized in the spleen, liver, bone marrow, kidneys and lungs. 70–80% of these are broken down by reticuloendothelial (RE) cells and excreted in the urine.

Production of antibodies

Antigen processing and presentation

Antigens are presented to **immunocompetent cells (ICCs)** by antigen-presenting cells (APCs) (macrophages and dendritic cells). With many antigens (T cell dependent antigens such as proteins and erythrocytes), processing by macrophages is prerequisite for antibody formation. But for T cell independent antigens, such as polysaccharides, antibody production does not require T cell participation. APC can ingest antigen, degrade it and present it to T cell. T cell is able to recognize only when the processed antigen is presented on the surface of APC, in association with MHC molecules to the T cell carrying the T cell receptor (TCR) for the epitope.

The antigen has to be presented complexed with MHC class II in case of CD4 (helper T/Th) cells, and for CD8 (cytotoxic T/Tc) cells with MHC class I molecules.

T and B cell activation

The activation of Th cell requires two signals for activation. The first signal is a combination of the TCR with the MHC class II-complexed antigen. The second signal is interleukin-1 (IL-1) which is produced by the APC. The activated Th cell produces IL-2 and other cytokines required for B cell stimulation. These include IL-4, IL-5 and IL-6 which act as B cell growth factor (BCGF) and B cell differentiation factor (BCDF). They activate B cells which have combined with their respective antigens to clonally proliferate and differentiate into antibody secreting plasma cells. A small proportion of B cells, instead of being transformed into plasma cells, become long-lived memory cells producing a secondary type of response to subsequent contact with the antigen. B cells carry surface receptors which consist of IgM or other immunoglobulin classes. A plasma cell secretes an antibody of a single specificity of a single antibody class (IgM, IgG or any other single class). However, in primary humoral response, plasma cells secrete IgM and later switch over to form IgG.

CD8 (cytotoxic T/Tc) cells are activated when they come into contact with antigens presented along with MHC class I molecules. They also need a second signal IL-2, which is secreted by activated Th cells. On contact with a target cell carrying the antigen on its surface, the activated Tc cells release cytokines that destroy the target, which may be virus infected or tumour cells. Some T cells also become memory cells.

Theories of antibody production

These fall into two groups:

1. Instructive theories.
2. Selective theories.

Instructive theories postulate that an ICC is capable of synthesizing antibodies of all specificities. An antigen encounters an ICC and instructs it to produce the complementary antibody.

On the other hand, selective theories postulate that ICCs have only restricted immunological range. The antigen selects its ICC and stimulates it to synthesize its antibody.

Instructive theories

1. Direct template theories

These theories were proposed by Breinl and Haurowitz (1930), Alexander (1931), and Mudd (1932). According to these, the antigen or the antigenic determinant enters the antibody-forming cell and serves as a template against which antibody molecules are synthesized so that they have combining sites complementary to the antigenic determinants.

2. Indirect template theory

This theory was proposed by Burnet and Fenner (1949). According to this theory, antigenic determinant enters into the ICC and gets incorporated in its genome and transmitted to the progeny cells. This theory, therefore, explained the specificity and the secondary response.

Selective theories

1. Side chain theory

This theory was proposed by Ehrlich (1898). According to this theory, ICCs have surface receptors which are capable of reacting with antigens which have complementary side chains. When foreign antigens are introduced into the body, they combine with those cell receptors which have a complementary fit. This inactivates the receptors and interferes with absorption of nutrients. As a compensatory mechanism, there is an overproduction of the same type of receptors which spill over into the blood as antibodies.

2. Natural selection theory

This theory was proposed by Jerne (1955). This theory postulates that about one million globulin molecules are formed in embryonic life, which covers full range of antigens. When an antigen is introduced, it combines with the globulin molecule that has the nearest complementary fit. The globulin with the combined antigen enters into antibody forming cells and stimulates them to produce same kind of antibody.

3. Clonal selection theory

This theory was proposed by Burnet (1957). This theory states that during immunological development a large number of lymphocytes capable of reacting with different antigens are formed. Cells with immunological reactivity with self-antigens are eliminated during embryonic life. Such clones are known as **forbidden clones**. *Their persistence or development in the later life leads to autoimmunity*. Each ICC is capable of reacting with one antigen. Contact with specific antigen leads to cellular proliferation to form clones which synthesize antibodies. This theory is more widely accepted than other theories.

Monoclonal antibodies

Principle

If an antigen is injected into an animal, the latter produces different types of antibodies against various epitopes of the

antigen. The antibodies thus generated are polyclonal in nature. This means different clones of antibody secreting cells are simultaneously synthesizing the antibody. Different molecules will have different specificities and affinities. In all microbial infections, body reacts with polyclonal antibody production. When these polyclonal antisera are used in bacterial test systems, **cross reactivity** often occurs.

A single antibody-forming cell or clone produces antibodies directed against specific epitope of the antigen. Such antibodies produced by a single clone and directed against a single antigenic determinant are called **monoclonal antibodies (MCA)**. In nature, MCA are produced in multiple myeloma where only one clone secretes a particular type of antibody. MCA can be generated in the laboratory. The theory of MCA production is based on clonal selection hypothesis of Burnet (1959) and the method for production of MCA was described by Kohler and Milstein in 1975, for which they were awarded Nobel Prize for Medicine in 1984.

The main breakthrough was not that a single line of monoclonal antibody producing cells could be isolated, but rather that the mouse splenic lymphocytes could be fused with mouse myeloma cells to produce **hybrid cells (hybridoma)**. Among the two cell types chosen for fusion, one provides the hybrid cell immortality (**myeloma cell**) while the other (**splenic plasma cell**) provides the antibody producing capacity. Such hybridomas can be maintained indefinitely in culture and continue to form MCA.

Technique

- Lymphocytes from the spleen of a mouse immunized with desired antigen are fused with mouse myeloma cells grown in culture which is deficient in the enzyme hypoxanthine phosphoribosyl transferase (HPRT) (Fig. 15.2).
- The fused cells are placed in a basal culture medium containing hypoxanthine, aminopterin and thymidine (HAT medium).
- Only hybrid cells possessing properties of both the splenic lymphocytes (HPRT+) and myeloma cells (HPRT-) can grow in culture. Normal lymphocytes cannot replicate indefinitely and unfused myeloma cells are killed by the aminopterin in HAT medium.
- The peritoneal cavity of mice, preferably of the same strain that was used for initial immunization step, can be used to grow the selected hybrid cell clone. First the peritoneal cavity is injected with an organic irritant such as pristane to produce chemical peritonitis. Next the selected hybrid cell line is injected into the peritoneal cavity. Within days, a tumour known as hybridoma develops. This tumour produces large quantities of MCA that can be harvested by aspirating ascitic fluid from mouse's peritoneal cavity.
- A tumour-bearing mouse will survive for 4–6 weeks, during which time large quantities of antibody can be recovered.
- Hybridomas can also be grown in tissue cultures where highly purified antibodies are produced without contamination from serum, ascites proteins or the cross reactivity of histocompatibility antibodies derived from mouse tissues.

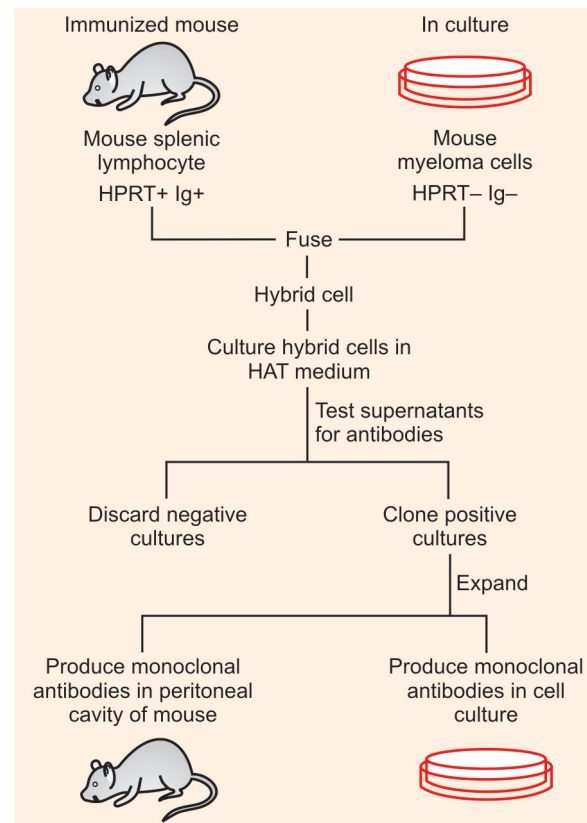


Fig. 15.2: Production of monoclonal antibodies.

Applications of monoclonal antibodies

Monoclonal antibodies have been produced for specific epitopes of a wide variety of viruses, bacteria (including mycobacteria), parasites and fungi. Many of the commercial systems using direct fluorescence and enzyme-linked assays utilize monoclonal antibody conjugates.

Adjuvants

Adjuvants are compounds that potentiate the immune response when mixed and administered with antigens. They contribute to greater and more prolonged antibody production and increased effector cell counts. They act in several ways:

1. Sustained release of antigen from the depot.
2. Stimulate lymphocytes non-specifically.
3. Activate macrophages.

Types of adjuvants

- **Depot:** A number of substances such as aluminium hydroxide or phosphate and an aqueous solution of an antigen may be emulsified in light mineral oil so that tiny water droplets containing antigen are dispersed throughout the oil (water-in-oil emulsion). The emulsion forms a depot of antigen in the tissues from which small quantities of antigen are released slowly sometimes for a year or more. This is known as **Freund's incomplete adjuvant**.
- **Complete Freund's adjuvant:** It possesses killed mycobacteria in addition to incomplete adjuvant. Besides increasing humoral immune response, it induces high degree of cellular immunity (delayed hypersensitivity) as

well. However, this adjuvant cannot be used in humans, because it produces local granuloma, and must be replaced by other adjuvants such as alum or killed *Bordetella pertussis*.

Immunosuppressive agents

Immunosuppressive agents are those which inhibit the immune response of macrophages, and B and T lymphocytes leading to lowered capacity to phagocytosis or to produce immunoglobulins and lymphokines. Organ and bone marrow transplantation became practicable on a large-scale only after powerful immunosuppressive drugs became available. They may also be used to control autoimmune responses or to prevent allergic reactions.

A wide range of physical, chemical and biological agents have been tried (Table 15.1). Some of these were soon dropped because the margin of safety was too small and toxic effects too pronounced.

Table 15.1: Immunosuppressive agents

Physical agents

- X-irradiation
- Surgery

Chemical agents

- Alkylating agents
 - Cyclophosphamide
 - Nitrogen mustard
- Corticosteroids
- Antimetabolites
 - Folic acid antagonists (methotrexate)
 - Analogues of purine (6-mercaptopurine and azathioprine)
 - Cytosine
 - Uracil (5-fluorouracil)
- Miscellaneous
 - Cyclosporine

Biological agents

- Antilymphocytic serum

- **X-irradiation** is more cytotoxic to replicating cells and has been used to prolong transplant survival and to control certain autoimmune conditions.
- Corticosteroids and cyclosporine impair maturation of activated cells by suppressing production of interleukins (ILs). Corticosteroids are antiinflammatory drugs that diminish the responsiveness of both B and T cell pools. They can inhibit production of IL-1 and IL-2. Prolonged use of corticosteroids may lead to hypertension, bone necrosis, cataract and mental disturbances.
- Cyclosporine has been widely used as an immunosuppressant in organ transplantation. It causes immune suppression by inhibiting the production of IL-2. It may have adverse side-effects affecting the liver and kidney.
- **Cytotoxic drugs**, such as azathioprine or cyclophosphamide, act on various stages of nucleic acid synthesis preventing replication of active lymphocytes. Azathioprine, used in renal transplant, inhibits T cells, but not B cell responses. Cyclophosphamide, used in bone marrow recipients, selectively prevents B cell replication.

T cell population can be depleted by use of antilymphocytic serum produced in horses. This destroys body's T cell pool but leaves antibody production intact. Unfortunately, it also reduces the ability of the body to fight viral infection.

CELL-MEDIATED IMMUNE RESPONSES

Cell-mediated immunity (CMI) normally refers to specific acquired immunity, which is accomplished by effector T cells and macrophages rather than B cells and antibodies. This includes allograft rejection, delayed hypersensitivity (DH) and cytotoxic reactions against intracellular parasites. As in case of antibody-mediated immune response, cell-mediated immune response can also be divided into primary and secondary cell-mediated immune responses.

Primary cell-mediated immune response

This is produced by initial contact with a foreign antigen. Foreign antigen is presented by antigen-presenting cells (APCs) to T cells leading to their activation. T cells possess antigen recognition receptors known as T cell receptors (TCRs) that recognize foreign antigen and a self-MHC molecule on the surface of the APC (Fig. 15.3). Because of the specificity of the TCRs only particular cells become activated. These cells proliferate and produce specific clones of effector T cells (Th, Tc, Td and Ts). Cell-mediated immune response develops after several days of antigenic challenge.

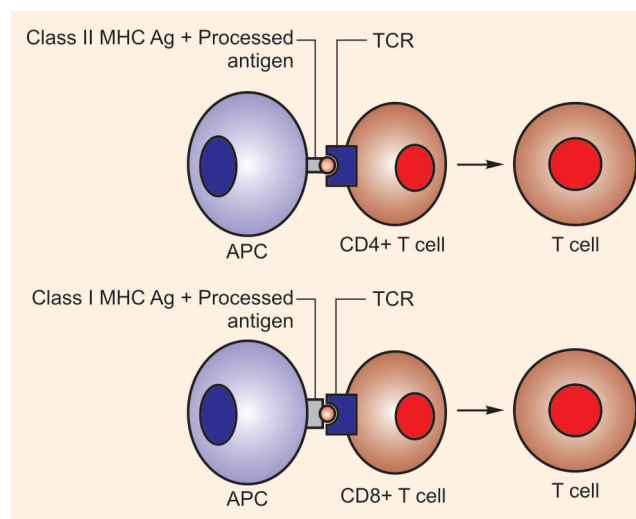


Fig. 15.3: Cell-mediated immune response.

Secondary cell-mediated immune response

If the same host is subsequently exposed to the same antigen, then the secondary cell-mediated immune response is usually more pronounced and occurs more rapidly. Because of the availability of specific memory cells, an increased number of effector cells are produced.

T cell differentiation

APCs, such as macrophages, phagocytose the antigen and degrade it. Subsequently, portions of antigen become

associated with MHC antigens and are expressed on APCs surface. Two modes of processing are known (Fig. 15.3):

- One mode is seen in case of processing of phagocytosed material such as bacteria. The antigenic material dissociated from the bacteria is associated with class II MHC molecules probably within the phagosome. MHC-antigen complex then expresses on the surface of the APC.
- Second mode is seen in processing antigens derived within the cell, for example, viral antigens synthesized in infected cell. However, this antigenic determinant associates with class I MHC molecule probably in the endoplasmic reticulum. MHC-antigen complex then expresses on the surface of the APC.

CD8⁺ cells recognize the combination of foreign antigen and class I MHC antigen and differentiate into Tc and Ts lymphocytes while CD4⁺ cells recognize the combination of antigen and class II MHC antigen and differentiate into Th and Td cells.

Lysis of target cell

Tc cell recognizes foreign antigen and class I MHC antigen and gets attached to the target cell expressing these on their

surface. This stimulates Tc cells to release **cytolysins**. This leads to calcium-dependent lysis of the target cell. Subsequently, the Tc cell can detach from the target cell and repeat this process with another. Recognition of target cells also stimulates Tc cells to synthesize and secrete **interferon- γ** , and thus they probably also contribute to some extent to macrophage activation.

Cytokines

These are biologically active substances produced by cells that influence other cells. They are referred to as **lymphokines** if they are derived from lymphocytes; and **monokines**, if they are derived from monocytes and macrophages. Interleukins are a family of cytokines that function primarily as growth and differentiation factors. Cytokines have been named based on the biological effects they produce. Various cytokines are given in Table 15.2.

Detection of CMI

Development of CMI can be detected by following methods.

1. Skin test for DH.
2. Transformation of cultured sensitized T lymphocytes on contact with antigen.

Table 15.2: Source and activity of cytokines

Cytokine	Source	Activity
Macrophage stimulating factor (MSF)	Td cells	Stimulates macrophage migration to the site of action
Macrophage activating factor (MAF)	Td cells	Restricts macrophage movement and increases phagocytic activity
Migration inhibiting factor (MIF)	Td cells	Inhibits migration of macrophages
Chemotactic factor (CF)	Td cells	Stimulates chemotaxis of macrophages
Interferon-gamma (IFN- γ)	Th, Td and NK cells	Increases cytotoxicity of NK cells and macrophages
Interleukin-1 (IL-1)	NK cells, APCs, B cells and T cells	Promotes growth and expression of fibroblasts, NK cells, B cells and T cells
Interleukin-2 (IL-2)	Th cells	Promotes B cell differentiation and T cell growth
Interleukin-3 (IL-3)	Th cells	Acts as a growth factor for bone marrow stem cell
Interleukin-4 (IL-4)	Th cells	Acts as a growth factor for macrophages, mast cells, B cells and T cells
Interleukin-5 (IL-5)	Th cells	Promotes B cell growth, antibody production and maturation of eosinophils
Interleukin-6 (IL-6)	Macrophages	Promotes B cell growth
Interleukin-7 (IL-7)	Bone marrow stroma cell	Stimulates B cell and T cell proliferation
Interleukin-8 (IL-8)	Mononuclear cells, endothelial cells and skin fibroblasts	Stimulates chemotaxis of neutrophils and T cells
Interleukin-9 (IL-9)	Activated T cells	Stimulates proliferation of IL-3 dependent myeloid cells and mast cells
Interleukin-10 (IL-10)	Th cells	Inhibits production of IFN and mononuclear cell functions
Interleukin-11 (IL-11)	Bone marrow and stromal cells	Induces acute phase proteins
Interleukin-12 (IL-12)	T cells	Activates natural killer (NK) cells
Interleukin-13 (IL-13)	T cells	Inhibits mononuclear cell functions
Interleukin-14 (IL-14)	T cells	Stimulates proliferation of activated B cells, inhibits immunoglobulin production
Interleukin-15 (IL-15)	Monocytes	Proliferation of T cells and activated B cells
Interleukin-16 (IL-16)	Eosinophils, CD8 ⁺ T cells	Chemoattraction of CD4 ⁺ T cells
Interleukin-17 (IL-17)	CD4 ⁺ T cells	Release of IL-6, IL-8
Interleukin-18 (IL-18)	Hepatocytes	Induces production of interferon- γ , enhances NK cell activity

3. Target cell destruction: Killing of cultured cells by T lymphocytes sensitized against them.
4. Migration inhibiting factor (MIF) test (Fig. 15.4).

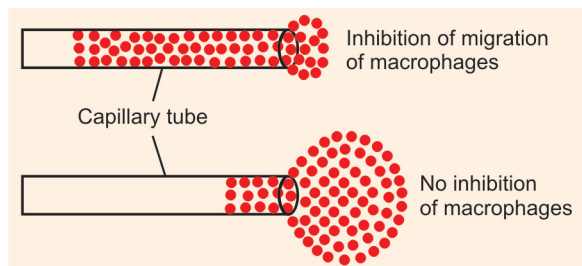


Fig. 15.4: Migration inhibiting factor test.

MIF test is most commonly employed. If a piece of capillary tube containing peritoneal exudate cells (macrophages and a few lymphocytes) is placed in a tissue culture chamber containing tissue culture fluid and no antigen, the macrophages migrate out of the open end of the tube into culture fluid to form a fan-like pattern. However, if the macrophages are obtained from a guinea pig sensitized to tuberculo-protein, addition of tuberculin to the culture chamber will inhibit migration.

Transfer factor

Lawrence (1954) reported transfer of CMI in man by injection of extract from the leucocytes from immunized individual. The extract from the leucocytes contains a soluble factor called transfer factor (TF). The transferred immunity is specific in that CMI can be transferred only to those antigens to which the donor is sensitive.

TF is a nucleopeptide with a molecular weight of 2,000–4,000 daltons. It is non-antigenic. It is resistant to trypsin but gets inactivated by heating at 56°C in 30 minutes. The mode of action of TF is not known. It appears to stimulate the release of lymphokines from sensitized T lymphocytes. It does not promote antibody synthesis. TF has been used in patients with:

- T cell deficiency (Wiskott-Aldrich syndrome).
- Disseminated infections associated with deficient CMI (lepromatous leprosy, tuberculosis and mucocutaneous candidiasis).
- Malignant melanoma and other types of cancer.

Immunological tolerance

Immunological tolerance may be defined as a state of unresponsiveness to specific antigens. This unresponsiveness is specific to antigens to which the individual is tolerant. Response to other antigens is unaffected. Two forms of tolerance can be identified—natural tolerance and acquired tolerance.

Natural tolerance

It is non-responsiveness to self-antigens. This arises during foetal development, when the immune system is being formed and maturing. If this tolerance breaks down and body responds to self molecules then an autoimmune disease will develop. Dizygotic cattle twins, which are genetically dissimilar, share the same placental circulation *in utero*. As adults, each twin fails to mount immune response to histocompatibility antigens on the cells of the other twin. Thus, they accept transplants

from each other. This could be accounted for by induction of specific immunological tolerance during foetal life. Based on this observation of Owen (1945), Burnet and Fenner (1949) suggested that the unresponsiveness of individuals to self-antigens was due to the contact of the immature immunological system with self-antigens during embryonic life.

Any antigen that comes into contact with the immunological system during its embryonic life would be recognised as a self-antigen and would not induce any immune response. They postulated that tolerance could be induced against foreign antigens, if they were administered during embryonic life. Medawar and his colleagues (1953) proved it experimentally. When skin graft from one inbred strain of mice (CBA) is applied on a mouse of another strain (A), it is rejected. But if CBA cells are injected into foetal or newborn A strain mice, the latter when grow up will freely accept skin grafts from CBA mice.

Certain strains of mice that are genetically deficient in the C5 complement component make vigorous antibody response when immunized with pure C5 taken from normal animals. Normal animals (which are not deficient in C5) do not respond to similar immunization.

Acquired tolerance

It arises when a potential immunogen induces a state of unresponsiveness to itself. This has consequences for host defences since the presence of a tolerogenic epitope on a pathogen may compromise the ability of the body to resist infection. For acquired tolerance to be maintained the tolerogen must persist or be repeatedly administered. This is probably necessary because of the continuous production of new B and T cells that must be rendered tolerant.

A number of factors influence the induction of tolerance. These include species and immunocompetence of the host, and physical nature, dose, and route of administration of antigen. Rabbits and mice can be rendered tolerant more rapidly than guinea pigs and chickens. Higher the degree of immunocompetence of the host, the more difficult it is to induce tolerance. Therefore, embryos and newborns are particularly susceptible for induction of tolerance.

It is easier to induce tolerance to a soluble macromolecule than to an aggregated antigen. For example, when human gammaglobulin is heat aggregated, it is highly immunogenic in mice, but when de-aggregated it is tolerogenic. This is probably due to the fact that aggregated antigens are readily phagocytosed by macrophages, where they can be presented to antibody-forming cells thus inducing antibody synthesis. On the other hand, soluble antigens are not so easily processed and may be more effective in inducing the Ts suppressor circuit.

The induction of tolerance is dose-dependent. Generally, high doses of antigen tolerate B cells, while minute doses given repeatedly tolerate T cells. A moderate dose of the same antigen might be immunogenic. The route of administration is also important. Intravenously administered antigens have faster contact with more cells at the highest concentration of tolerogen. Moreover, an intravenous injection rapidly reaches the spleen to which Ts cells migrate leading to tolerance.

Tolerance can be overcome spontaneously or by injection of cross reacting immunogens. For example, tolerance to bovine serum albumin in rabbits can be abolished by immunization with cross reacting human serum albumin.

Mechanism of tolerance

Tolerance can arise through three possible mechanisms:

1. **Clonal deletion:** In embryonic life clones of B and T cells, possessing receptors that recognize self-antigens, are selectively deleted or eliminated and, therefore, no longer available to respond upon subsequent exposure to that antigen. This is known as clonal deletion.
2. **Clonal anergy:** Clones of B and T cells expressing receptors that recognize self-antigen might remain but they cannot be activated. This is known as clonal anergy.
3. **Suppression:** Clones of B and T cells expressing receptors that recognize self-antigens are preserved. Antigen recognition might be capable of causing activation, however, expression of immune response might be inhibited or blocked through active suppression.

KEY POINTS

- After **first injection of the antigen** there is a long **lag phase** of several days before **antibodies appear**.
- **Second contact** with antigen **stimulates** the pool of **memory cells** to produce a **larger and faster response** than primary reaction.
- Unlimited expansion of clones is restricted by antigen concentration and antibody feedback T cell suppression.
- **Cell-mediated immunity** refers to specific acquired immunity, which is accomplished by **effector T cells** and **macrophages**.
- **Cytokines** are biologically active substances produced by cells that influence other cells.
- Reactivity to self is prevented by a variety of **tolerance mechanisms**.



Important Questions

1. Discuss primary and secondary humoral responses.
2. What are monoclonal antibodies? How are they produced and what are their applications?
3. Write short notes on:
 - (a) Adjuvants
 - (b) Immunosuppressive agents
 - (c) Cytokines
 - (d) Burnet's clonal selection theory
4. What is immunological tolerance? Discuss various mechanisms by which tolerance can be induced.



Multiple Choice Questions

1. CMI participates in:
 - (a) allograft rejection.
 - (b) graft versus host reaction.
 - (c) delayed hypersensitivity reaction.
 - (d) all of the above.
2. Most widely accepted theory of antibody production is:
 - (a) direct template theory.
 - (b) indirect template theory.
 - (c) side chain theory.
 - (d) clonal selection theory.
3. Immunosuppressive agent/s is/are:
 - (a) methotrexate.
 - (b) cyclosporine.
 - (c) anti-lymphocytic serum.
 - (d) All of the above.
4. Delayed-type hypersensitivity cells secrete which of the following lymphokine/s?
 - (a) Migration inhibiting factor.
 - (b) Macrophage activating factor.
 - (c) Macrophage stimulating factor.
 - (d) all of the above.
5. Interleukin-2 promotes:
 - (a) B cell differentiation.
 - (b) bone marrow stem cells.
 - (c) maturation of eosinophils.
 - (d) growth of fibroblasts.
6. Interferon- γ is produced by following cells, **except**:
 - (a) Td cells.
 - (b) Th cells.
 - (c) macrophages.
 - (d) NK cells.
7. CMI can be detected by the following methods:
 - (a) Skin test for delayed hypersensitivity.
 - (b) Migration inhibition factor test.
 - (c) Target cell destruction.
 - (d) All of the above.
8. Mechanism/s of induction of tolerance is/are:
 - (a) clonal deletion.
 - (b) clonal anergy.
 - (c) suppression.
 - (d) all of the above.
9. Fusion between a plasma cell and a tumour cell produces:
 - (a) hybridoma.
 - (b) lymphoma.
 - (c) myeloma.
 - (d) natural killer cell.
10. Monoclonal antibodies recognize a single:
 - (a) antigen.
 - (b) epitope.
 - (c) paratope.
 - (d) none of these.
11. Which of the following antibodies would most likely be found in body secretions such as tears, milk, saliva and mucus?
 - (a) IgA.
 - (b) IgG.
 - (c) IgD.
 - (d) IgM.



Answers

1. d 2. d 3. d 4. d 5. a 6. c 7. d 8. d 9. a 10. b 11. a

Immunodeficiency Diseases

Competency achievement: The student after reading the chapter should be able to:

MI1.10(c): Describe immunological mechanisms in immunological disorders in immunodeficiency states.

Immunodeficiency diseases are the conditions where the defence mechanism of the body is impaired leading to repeated microbial infections and sometimes enhanced susceptibility to malignancies. These include defects in all components of immune system, including B, T and NK lymphocytes, phagocytic cells and complement proteins. Patients with defects in immunoglobulins, complement proteins or phagocytosis are very susceptible to recurrent infections with encapsulated bacteria such as *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Staphylococcus aureus*. These are called **pyogenic infections**, because the bacteria give rise to pus formation. On the other hand, patients with defects in cell-mediated immunity, i.e. in T cells, are susceptible to overwhelming, even lethal, infections with microorganisms that are ubiquitous in the environment and to which normal people rapidly develop resistance. For this reason these are called **opportunistic infections**.

Immunodeficiencies may be classified as primary or secondary.

- **Primary immunodeficiency** is due to the abnormalities in the development of the immune mechanisms.
- **Secondary immunodeficiencies** are secondary to some other disease process affecting the normal functioning of some part of the lymphoid tissues, drugs, malnutrition and ionizing radiations used to treat cancer.

Since 1981, the most common immunodeficiency disease is acquired immunodeficiency syndrome (AIDS).

PRIMARY IMMUNODEFICIENCY SYNDROMES

Attempts have been made to classify primary immunodeficiency disorders. Table 16.1 lists well-known primary immunodeficiency syndromes.

Table 16.1: Primary immunodeficiency syndromes

A. Humoral immunodeficiencies (B cell defects)

- X-linked (congenital) agammaglobulinaemia (Bruton's disease)
- Transient hypogammaglobulinaemia of infancy
- Common variable immunodeficiency
- Selective IgA deficiency
- Selective IgM deficiency
- Immunodeficiency with elevated IgM

B. Cellular immunodeficiencies (T cell defects)

- Thymic hypoplasia (DiGeorge's syndrome)
- Purine nucleoside phosphorylase (PNP) deficiency

C. Combined immunodeficiencies (B and T cell defects)

- Cellular immunodeficiency with abnormal immunoglobulin synthesis (Nezelof's syndrome)
- Ataxia telangiectasia
- Immunodeficiency with thrombocytopenia and eczema (Wiskott-Aldrich syndrome)
- Immunodeficiency with thymoma
- Severe combined immunodeficiency diseases
 - (a) Swiss-type agammaglobulinaemia
 - (b) Adenosine deaminase deficiency
 - (c) Reticular dysgenesis

D. Complement immune deficiency disorders

E. Disorders of phagocytosis

- Chronic granulomatous disease
- Myeloperoxidase (MPO) deficiency
- Leucocyte G6PD deficiency
- Chediak-Higashi syndrome

A. Humoral immunodeficiencies (B cell defects)

X-linked (congenital) agammaglobulinaemia (Bruton's disease)

This is the first immunodeficiency disease to have been recognised. It was described by Bruton in 1952. As an X-linked disease, it is seen almost entirely in males, but sporadic cases have also been described in females. It is characterized by recurrent infections with pyogenic bacteria, such as pneumococci, streptococci, staphylococci and *Haemophilus*.

The basic defect in this disorder is a failure of pre-B cells to differentiate into mature B cells. Tonsils and adenoids are

atrophic. Lymph node biopsy reveals a depletion of cells of bursa-dependent areas. Plasma cells and germinal centres are absent even after antigenic stimulation. B cells are absent or remarkably decreased in the circulation and there is an absence or deficiency of all the five classes of serum immunoglobulins. Pre-B cells are found in normal numbers in bone marrow. These diseases can be treated by routine injections of normal immunoglobulins.

Transient hypogammaglobulinaemia of infancy

This is due to an abnormal delay in the initiation of IgG synthesis in some infants. It involves infants of both sexes and is not a common entity. Normally, infants start producing their own antibodies by 3–7 months, but in infants with this condition antibody production starts by 6–11 months of age. Lymphocytes show no abnormality in the percentage of cells in different subpopulations. Gammaglobulin therapy is not indicated in this condition, as it may contribute to prolongation of immunodeficiency by a negative feedback inhibition of IgG synthesis.

Common variable immunodeficiency

Common variable immunodeficiency is also known as late onset hypogammaglobulinaemia because it usually manifests only by 15–35 years of age. The patient may have normal-sized or enlarged tonsils, lymph nodes and spleen. The serum immunoglobulin and antibody deficiency are usually as profound as in X-linked disorder and the kinds of infections experienced and bacterial etiologic agents involved are generally the same for the two defects. This may also be associated with an increased incidence of autoimmune disease. B cells may be present in circulation in normal numbers, but they appear defective in being unable to differentiate into plasma cells and secrete immunoglobulins. Thus, this defect appears to be caused by abnormal terminal differentiation of the B cell line. The total immunoglobulin level is usually less than 300 mg per 100 ml, with IgG less than 250 mg per 100 ml. Increased suppressor T cell and decreased helper T cell activity has been proposed as a cause of this disorder. Treatment of this condition is by administration of gammaglobulin preparations intramuscularly or intradermally.

Selective IgA deficiency

Absence or near absence of serum and secretory IgA is thought to be the most common well-defined immunodeficiency disorder. Because of the deficiency of secretory IgA, infections occur predominantly in the respiratory, gastrointestinal and urogenital tracts. Serum concentration of other immunoglobulins are usually normal in patients with selective IgA deficiency. Anti-IgA antibodies are present in the sera of nearly half of the patients with selective IgA deficiency and administration of IgA-containing solutions to these patients may lead to severe or fatal anaphylactic reactions.

Selective IgM deficiency

Selective IgM deficiency may occur occasionally. These patients develop septicaemia due to meningococci and other Gram-negative bacteria, pneumococcal meningitis, recurrent

staphylococcal pyoderma, periorbital cellulitis, bronchiectasis, otitis and other respiratory infections.

Immunodeficiency with elevated IgM

This disorder is characterized by low IgA and IgG levels with either a normal or more frequently with elevated IgM. Patients with this defect may develop recurrent pyogenic infections, including otitis media, sinusitis, pneumonia and tonsillitis during first or second year of life. In addition, patient may develop autoimmune disorders such as thrombocytopenia, neutropenia and haemolytic anaemia. Some patients develop malignant infiltration with IgM producing cells. T cell functions, in these patients, are usually normal.

B. Cellular immunodeficiencies (T cell defects)

Thymic hypoplasia (DiGeorge's syndrome)

Thymic hypoplasia results from a congenital malformation affecting the third and fourth pharyngeal pouches. These structures give rise to the thymus, parathyroid glands, and portions of lips, ears and the aortic arch. Therefore, in addition to thymic hypoplasia the parathyroid glands are also either hypoplastic or totally absent. The functions of both these organs are essential for survival, the thymus for the formation of competent T cells to carry out cellular immune functions and parathyroid for the production of the hormone, parathormone, which regulates calcium levels in the blood. Most of these infants have additional developmental defects affecting the face, ears, heart and great vessels.

In these patients thymus is usually rudimentary and T cells are deficient or absent in the circulation. They are similarly depleted in thymus-dependent areas of the lymph nodes and spleen. Thus, infants with this defect are extremely vulnerable to viral, fungal and protozoal infections. Susceptibility to intracellular bacteria is also increased, because phagocytic cells that eliminate them require T cell-derived signals for activation. The B cell system and serum immunoglobulins are entirely unaffected.

Purine nucleoside phosphorylase (PNP) deficiency

The lack of enzyme PNP because of a gene defect in chromosome 14 results in impaired metabolism of cytosine and inosine to purine. These patients show decreased T cell proliferation leading to decreased T cell-mediated immunity and recurrent or chronic infections.

C. Combined immunodeficiencies (B and T cell defects)

Cellular immunodeficiency with abnormal immunoglobulin synthesis (Nezelof's syndrome)

In this condition there is depressed cell-mediated immunity associated with decreased, normal or increased levels of most of the five immunoglobulin classes. Patients present during infancy with recurrent or chronic pulmonary infections, failure to thrive, oral or cutaneous candidiasis, chronic diarrhoea, recurrent skin infections, Gram-negative sepsis, urinary tract infections and severe varicella. Other findings include lymphopenia, neutropenia and eosinophilia. The thymus is small and peripheral lymphoid tissues are hypo-

plastic and demonstrate paracortical lymphocyte depletion. Bone marrow transplantation, thymus transplantation and transfer factor have been used for treatment with success in some cases. For the treatment of microbial infections adequate antimicrobial therapy is essential.

Ataxia telangiectasia

Ataxia telangiectasia is a multisystem defect involving vascular, endocrine, nervous and immune systems. Clinically, patient develops cerebral ataxia, oculocutaneous telangiectasia, chronic sinopulmonary disease, ovarian dysgenesis, a high incidence of malignancy and variable humoral and cellular immunodeficiency. Mental capacity, immune status and other physiological conditions deteriorate with time. The majority of patients lack IgA and IgE antibodies. Some patients possess antibodies to IgA. Cell-mediated immunity is also defective leading to impairment of delayed hypersensitivity and graft rejection. Severe infections begin to occur during the first year of life. Death occurs due to sinopulmonary infections early in life, or malignancy in the second or third decade.

Immunodeficiency with thrombocytopenia and eczema (Wiskott-Aldrich syndrome)

Wiskott-Aldrich syndrome is an X-linked recessive disease characterized by bleeding, eczema and recurrent infections. The bleeding is due to thrombocytopenia, eczema from elevated IgE levels and recurrent bacterial, viral and fungal infections from abnormalities in cell-mediated (thymic hypoplasia) and antibody-mediated (lymphoid hyperplasia) immunity. B and T cell numbers are normal initially, but by 6 years of age there is a profound loss of cell-mediated immunity. Transfer factor and foetal thymus transplants have been tried with some benefit.

Immunodeficiency with thymoma

This syndrome consists of thymoma with impaired cell-mediated immunity and agammaglobulinaemia. This usually occurs in adults. They may also have eosinophilia or eosinopenia, haemolytic anaemia, agranulocytosis, thrombocytopenia and pancytopenia. Antibody formation is poor and progressive lymphopenia develops. Several patients with this disorder have been shown to have excessive suppressor T cell activity.

Severe combined immunodeficiency diseases

Severe combined immunodeficiency diseases (SCID) include syndromes in which patients lack both B and T cells. Many distinct patterns of severe combined immunodeficiency have been described:

(a) Swiss-type agammaglobulinaemia

This is inherited either through a sex-linked pattern or as an autosomal-recessive trait. Such persons are born with stem-cell defects and lack both antibody and cell-mediated immunity. They have agammaglobulinaemia with lymphocytopenia.

(b) Adenosine deaminase deficiency

Certain types of immune deficiency disorders arise as a consequence of reduced enzymatic activity. One such example of a SCID has a genetically inherited enzyme deficiency disorder.

The enzyme that is deficient in this disorder is adenosine deaminase (ADA), an enzyme involved in purine metabolism. In these patients, T cell deficiency is more profound than B cell deficiency. ADA levels are low in all the tissues, including red blood cells. The mechanism by which this deficiency causes SCID is not clear. It is, however, believed that deficiency of this enzyme leads to accumulation of adenosine and deoxyadenosine triphosphate, which are toxic to lymphocytes, particularly of T cell lineage. Infants with these severe immune disorders are vulnerable to all forms of viral, fungal and bacterial infections, and most die during the first year of life.

(c) Reticular dysgenesis

This is the most serious form of SCID. Here the defect is in the development of the multipotent bone marrow stem cell. The individual is born without any white blood cells, neither phagocytes nor lymphocytes. This is known as reticular dysgenesis. As a result of this disorder there is the absence of immunologically competent cells. A baby with this disorder usually dies within the first year of life from recurrent, intractable infections.

D. Complement immune deficiency disorders

Deficiency of C1 and C4 is associated with systemic lupus erythematosus. In C2 deficiency, there appear to be few problems probably because the alternative pathway of complement can still be used. The absence of C3 component is associated with increased susceptibility to bacterial infections, such as pneumonia, meningitis and septicaemia.

Persons deficient in C5 to C8 can be asymptomatic for years and then develop infections caused by *Neisseria meningitidis* and *N. gonorrhoeae*, and immune-complex syndromes with rheumatoid-like symptoms. So far, there is no known disease with deficiency of C9.

E. Disorders of phagocytosis

Phagocytosis may be impaired either by intrinsic defects within the phagocytic cells such as enzyme deficiency or extrinsic defects due to deficiency of opsonic antibody, complement or due to drugs or antineutrophil antibodies. Phagocytic dysfunction leads to increased susceptibility to infections.

Chronic granulomatous disease

Chronic granulomatous disease (CGD) is a frequently fatal genetic disorder in which there is a deficiency of NADPH oxidase. Individuals with CGD possess polymorphonuclear leucocytes that phagocytose invading bacteria normally but are unable to kill many of the ingested microorganisms because engulfment of bacteria is not followed by activation of oxygen-dependent killing mechanisms. Many bacteria produce hydrogen peroxide, which can be utilized by neutrophils to form bactericidal compounds. However, bacteria like *Staphylococcus aureus* and those belonging to the family Enterobacteriaceae possess catalase, which inactivates hydrogen peroxide and thus prevents its utilization by the neutrophils. Against such bacteria the CGD neutrophils are defenceless. Therefore, CGD is characterized by repeated bacterial infections most commonly caused by these organisms.

Myeloperoxidase (MPO) deficiency

In this rare disease, leucocytes are deficient in MPO. MPO-deficient patients generally remain well and free from recurrent infections, however, they are liable to develop recurrent *Candida albicans* infection.

Leucocyte G6PD deficiency

In this rare disease leucocytes are deficient in glucose-6-phosphate dehydrogenase. These patients show diminished bactericidal activity after phagocytosis leading to repeated bacterial infections.

Chediak-Higashi syndrome

Chediak-Higashi syndrome (CHS) is an autosomal recessive disorder. The individuals with this syndrome suffer from recurrent infections similar to those seen in persons with CGD. Polymorphonuclear leucocytes in patients with CHS possess large (giant) lysosomes. These abnormal lysosomes do not fuse readily with cytoplasmic phagosome.

SECONDARY IMMUNODEFICIENCY

Acquired deficiencies of immunological mechanisms can occur secondarily to a number of disease states such as metabolic disorders, malnutrition, malignancy and infections or after exposure to drugs and chemicals. Secondary immunodeficiency is far more common than primary immunodeficiency.

Deficiency of immunoglobulins can be brought about by excessive loss of protein through diseased kidneys or via the intestine in protein-losing enteropathy. Malnutrition and iron deficiency can lead to depressed immune responsiveness, particularly in cell-mediated immunity. Irradiations, cytotoxic drugs and steroids often have undesirable effects on the immune system. Infections with human immunodeficiency, measles and other viruses are often immunosuppressive.

Raised immunoglobulin levels are seen in certain disorders of plasma cells due to malignant proliferation of a particular clone or group of plasma cells. In these conditions, such as chronic lymphocytic leukaemia and multiple myeloma each malignant clone will produce one particular type of antibody. This leads to decreased synthesis of normal immunoglobulins and increased susceptibility to bacterial infections. Cell-mediated immunity is depressed in lymphoreticular malignancies like Hodgkin's lymphoma, obstruction to lymph circulation and infiltration of the thymus-dependent areas of lymph nodes with non-lymphoid cells as in lepromatous leprosy.

Acquired immunodeficiency syndrome (AIDS), which has assumed pandemic proportion, is the most important of secondary immunodeficiency diseases (see Chapter 64).

KEY POINTS

- In **immunodeficiency disease** the defence mechanism of the body is impaired leading to **repeated microbial infections**.
- **Primary immunodeficiencies** are due to the abnormalities in the development of the immune mechanisms.
- **Secondary immunodeficiencies** are secondary to some other disease process affecting the normal functioning of some parts of lymphoid tissues, drugs, malnutrition and ionizing radiations used to treat cancer.
- Patients with T lymphocyte abnormalities have a higher incidence of oral disease than patients with B lymphocyte disorders have.
- Since 1981, the most common immunodeficiency disease is **acquired immunodeficiency syndrome (AIDS)**.

Important Questions

1. What are immunodeficiency diseases? Differentiate between primary and secondary immunodeficiencies.
2. List well-known primary immunodeficiency syndromes. Describe Bruton's disease.
3. Write short notes on:
 - (a) DiGeorge's syndrome
 - (b) Wiskott-Aldrich syndrome
 - (c) Chediak-Higashi syndrome

Multiple Choice Questions

1. Thymic hypoplasia leads to:
 - (a) humoral immunodeficiency.
 - (b) cellular immunodeficiency.
 - (c) complement deficiency.
 - (d) none of the above.
2. Myeloperoxidase deficiency is a defect of:
 - (a) B cells.
 - (b) T cells.
 - (c) complement.
 - (d) phagocytes.
3. Polymorphonuclear leucocytes in patients with which condition possess large lysosomes?
 - (a) Chediak-Higashi syndrome.
 - (b) Chronic granulomatous disease.
 - (c) Myeloperoxidase deficiency.
 - (d) Leucocyte G6PD deficiency.
4. Due to the deficiency of secretory IgA, infections occur predominantly in:
 - (a) respiratory tract.
 - (b) gastrointestinal tract.
 - (c) urogenital tract.
 - (d) all of the above.
5. The most common immunoglobulin deficiency is of:
 - (a) IgA.
 - (b) IgD.
 - (c) IgE.
 - (d) IgM.

Answers

1. b
2. d
3. a
4. d
5. a

Hypersensitivity

Competency achievement: The student after reading the chapter should be able to:

MI1.10(a): Describe the immunological mechanisms of hypersensitivity and discuss the laboratory methods used in detection of hypersensitivity.

PA9.2: Describe the mechanisms of hypersensitivity reactions.

Hypersensitivity is an abnormal immune response which produces physiological or histopathological damage in the host. It may be divided into five types:

- Type I Anaphylactic
- Type II Cytotoxic
- Type III Immune complex
- Type IV Cell-mediated or delayed
- Type V Stimulatory or antireceptor

Types I, II, III and V hypersensitivity depend on the interaction of antigen with humoral antibodies and are known as immediate type reactions, although some are more immediate than others. Immediate hypersensitivity reactions develop in less than 24 hours after re-exposure to an antigen. Type IV hypersensitivity or delayed hypersensitivity is mediated by T lymphocytes. Delayed hypersensitivity reactions develop in 24–48 hours.

TYPE I HYPERSENSITIVITY: ANAPHYLACTIC

It is **mediated by IgE antibody** and is due to the powerful effects of histamine and other vasoactive amines. Hypersensitivity may be local or generalized, depending upon the amount of histamine released, the site of its release and route of stimulating antigen. Generally, small amount of antigen administered to mucous membrane or skin will induce local anaphylaxis, whereas larger amounts may induce a generalized reaction and antigen administered systemically may cause generalized anaphylaxis. Local anaphylaxis is exemplified by such conditions as hay fever and asthma. Systemic anaphylaxis is a shock like condition that can occur in individuals who are intensely allergic to such things as bee venom, penicillin and horse serum.

An antigenic substance that can trigger the allergic state is known as **allergen**. It may be a protein or chemically complex low molecular weight substance. Most allergens are considered weakly immunogenic and most people do not respond to them adversely. However, an allergic person is often sensitive to several different allergens.

Mechanism of type I hypersensitivity

In order to produce type I hypersensitivity an individual must first come in contact with an antigen and produce IgE antibodies. These antibodies bind to mast cells and basophils (Fig. 17.1). Basophils are found in the circulation while mast cells (or fixed basophils) are located in lymphoid regions of respiratory tract, gastrointestinal tract, reproductive tract, skin and lining of blood vessels including capillaries. They have large number of vesicles containing pharmacologically potent compounds like histamine and serotonin.

Thus, **after first exposure** allergen-specific IgE is fixed to the mast cells and basophils, thereby sensitizing them. The part of the IgE molecule that binds to the surface of mast cells and basophils is the Fc portion. These cells possess high affinity receptors specific for Fc portion of IgE antibodies. Thus, Fab portion of IgE remains exposed. IgE antibodies can remain attached on these cells for up to 6 weeks. Such an individual is said to be **sensitized**.

After a second exposure the allergen travels to the mast cells and basophils, where it binds to antigen-binding site on IgE molecule. Antigen–antibody binding triggers the process of degranulation through which these cells explosively discharge its pharmacologically active agents. These include histamine, serotonin, bradykinin, slow-reacting substance of anaphylaxis (SRS-A), platelet-activating factors, eosinophil chemotactic factor of anaphylaxis and prostaglandins.

Histamine

Histamine is the most abundant and fastest acting. It induces smooth muscle contraction, release of mucus, vasodilation and increased capillary permeability. All these reactions can have profound effects. For example, excessive smooth muscle contraction and release of mucus in respiratory tract can close the air passages of trachea and bronchi, causing asphyxiation and death by suffocation.

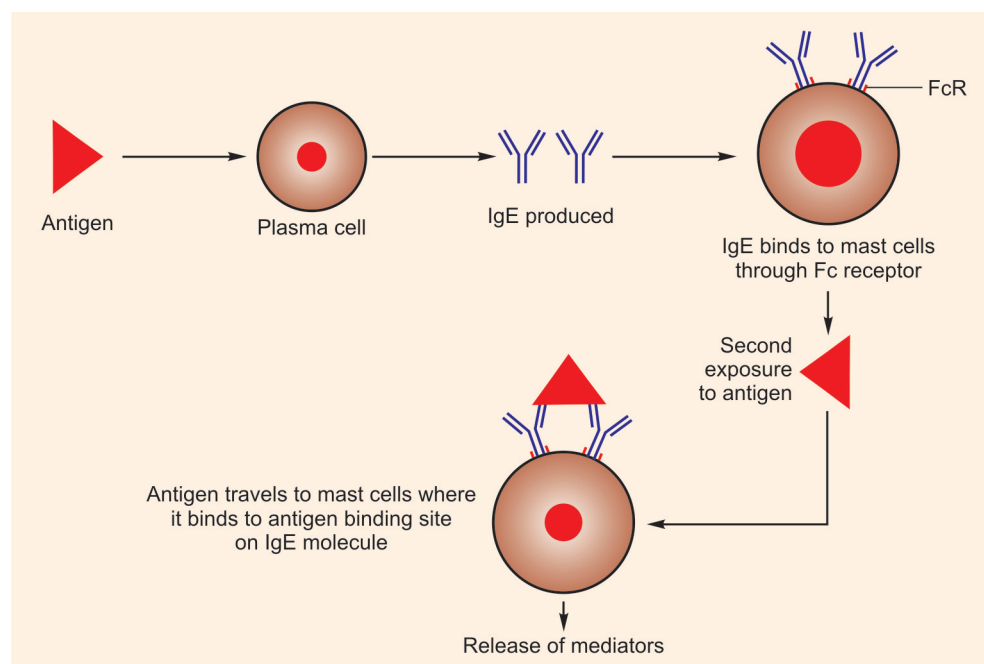


Fig. 17.1: Antigen-induced mediator release from mast cell.

Serotonin

This is a base derived by decarboxylation of tryptophan. It is found in the intestinal mucosa, brain tissue and platelets. It causes smooth muscle contraction, increased capillary permeability and vasoconstriction.

Eosinophil chemotactic factors of anaphylaxis

These are acidic tetrapeptides released from mast cell granules which are strongly chemotactic for eosinophils. These probably contribute to the eosinophilia accompanying many hypersensitivity states.

Slow-reacting substance of anaphylaxis

It is produced by leucocytes. It causes sustained constriction of smooth muscles and is much more potent bronchoconstrictor than histamine. It is not inhibited by antihistaminics.

Prostaglandins and leukotrienes

These are derived from arachidonic acid which is formed from disrupted cell membranes of mast cells and leucocytes. The lipoxygenase pathway leads to the formation of leukotrienes, while the cyclo-oxygenase pathway leads to prostaglandins and thromboxane. Prostaglandin $F2_{\alpha}$ and thromboxane $A2$ are powerful but transient bronchoconstrictors.

Platelet activating factor

It is a low molecular weight lipid released from basophils. It causes aggregation of platelets and release of their vasoactive amines.

Cutaneous anaphylaxis

If a person is suspected to be allergic to a particular substance, a **skin test** can be done. In this test a small dose of allergen

is injected intradermally. In less than 30 minutes results can be read. If the individual is allergic, there will be a **wheel and flare response** at the site of injection. Wheal is a pale central area of puffiness due to oedema (caused by increased capillary permeability) which is surrounded by flare (caused by hyperaemia due to vasodilation). Since the injection site is small, it is possible to test for hypersensitivity to hundreds of substances on a person's back. The wheal and flare response can be used to determine the specific substance to which the atopic person is sensitive.

Prausnitz-Küstner (PK) reaction

Prausnitz and Küstner in 1921 demonstrated transmission of IgE-mediated type I hypersensitivity by injecting serum containing IgE antibodies from allergic person into the skin of a normal or non-allergic person. Serum from Küstner, who was hypersensitive to certain species of cooked fish, was injected intracutaneously in Prausnitz (normal) followed 24 hours later by an intracutaneous injection of cooked fish, to which Küstner was sensitive, into the same site in Prausnitz. This led to wheal and flare reaction within 20 minutes. As IgE antibody is homocytotropic, the test has to be carried out on human skin, therefore, there is risk of transmission of hepatitis B virus and human immunodeficiency virus.

Passive cutaneous anaphylaxis (PCA)

PCA is similar to PK reaction. This is used to assay IgE antibodies in experimental animals. In this procedure, serum from an anaphylactically sensitized animal is injected intradermally into the skin of a normal animal. Forty-eight hours later, this animal is challenged with allergen (to which the first animal was sensitive) and Evans blue dye, injected intravenously. If IgE is present in the serum (from first animal), the allergen will bind to IgE on the mast cell and cause degranulation and the release of vasoactive amines.

The blood capillaries in the area become permeable and allow the Evans blue dye to leak out into the dermis of the skin leading to immediate blueing at the site of intradermal injection. The area of blueing can be related to the quantity of IgE present in the serum. PCA can be used to detect the human IgG antibody which is heterocytotropic (capable of fixing to cells of other species) but not IgE which is homocytotropic (capable of fixing to cells of homologous species only).

Anaphylaxis *in vitro* (Schultz-Dale phenomenon)

In 1910, Schultz and Dale demonstrated that sensitized strips of smooth muscle contract, *in vitro*, following exposure to antigen. Thus, intestinal and uterine muscle strips from a sensitized animal (guinea pig) held in Ringer's solution bath contract vigorously on addition of specific antigen. Smooth muscle strips can be passively sensitized by bathing them in serum from a hypersensitive animal. The actual test is done by adding the antigen and observing subsequent contractions.

Atopy

The term atopy refers to chronic human allergic states. These include **hay fever**, **allergic asthma**, **atopic dermatitis (urticaria)** and **food allergies**. The antigens commonly involved in atopy are inhalants (pollen, house dust, animal dander or other types of fine particles suspended in air) or ingestants (milk, milk products, eggs, meat, fish or cereal). Some of them are contact allergens, to which the skin and conjunctiva may be exposed. The mechanism of development of atopy is essentially the same as that of systemic anaphylaxis. Atopy is likely to develop when allergen is localized or absorbed slowly, on the other hand systemic anaphylaxis is likely to develop if large quantities of allergen are quickly distributed throughout the body.

Hay fever (allergic rhinitis)

It is an IgE-mediated allergic reaction that affects the mucosal surfaces of upper respiratory tract. It leads to nasal congestion, headache, running nose, watery eyes, itching and sneezing. The specific stimuli that cause allergic rhinitis include antigenic components of grass, weed and tree pollens, dust, mites, animal dander, organic dusts, components of tobacco smoke, and non-infectious components of fungal and bacterial allergens. If the allergen cannot be removed, then antihistaminics are the pharmacological agents of choice for the treatment of allergic rhinitis since histamine seems to be the major mediator of this allergy.

Allergic asthma

It is a severe form of respiratory allergy. It leads to contraction of the trachea and bronchi. The specific stimuli that cause allergic asthma include airborne allergens. In addition, asthma-producing foods such as milk, milk products, eggs, meat, fish or cereal may also precipitate allergic asthma. Some patients may even be sensitive to normal microbial flora leading to endogenous asthma. The main mediators of this allergy are serotonin and SRS-A. Therefore, antihistaminics

are not able to reverse the smooth muscle contraction. The drug of choice for the treatment of this allergy is adrenaline (epinephrine). It is dispensed as atomizers for quick delivery. In addition, corticosteroids and cromolyn sodium have proved beneficial in treating attacks of allergic asthma.

Atopic dermatitis (urticaria)

Allergic skin eruptions in the form of wheals (whitish swelling), hives (red lesions), or eczema (scaly sores) may develop from varied sources such as foods, drugs, chemicals and clothing materials. Avoidance of these is the treatment. If it is not possible then antihistaminics are indicated.

Food allergies

Food allergy occurs in infants and children, but is uncommon in adults. Certain foods such as eggs, milk, peanuts, seafoods, citrus fruits and chocolate are frequent causes of food allergy. Consumption of the food in a patient with food allergy leads to nausea, vomiting, diarrhoea and cramps with typical urticaria and wheezing or upper airways congestion. Best cure for food allergy is to avoid that food unless it is a common ingredient of prepared foods such as flour.

Desensitization

This is accomplished by actually injecting the person with offending allergen. The patient is injected with very small doses of allergen repeatedly over a long period of time (months). The dose is progressively increased until the person no longer reacts to the allergen. However, some persons may get relapse after years.

Following small earlier injections, the allergens react with IgE antibodies attached to mast cells and basophils leading to degranulation. This process goes on till all the IgE antibodies are consumed. Subsequently, in response to higher doses IgG antibodies are produced in place of IgE antibodies. IgG antibodies are known as **blocking antibodies** because they are able to bind to the allergen in the bloodstream before they reach IgE attached to the mast cells thus blocking degranulation. Most subclasses of IgG (with the exception of IgG4) cannot bind to mast cells. Another theory states that this type of immunotherapy stimulates the expression of suppressor T cells which block IgE antibody production by B cells. Therefore, the level of IgE antibodies falls and symptoms disappear.

TYPE II HYPERSENSITIVITY: CYTOTOXIC

This involves the combination of IgG and IgM serum antibodies with foreign antigenic components on a cell surface. Alternatively, a free foreign antigen or hapten such as a drug or microbial product may be adsorbed onto a cell membrane, which subsequently combines with antibody. Two different antibody-dependent mechanisms are involved in this type of hypersensitivity.

1. Complement-mediated cytotoxicity

Some of the cytotoxic reactions are complement dependent. After the antibodies become membrane bound, complement

may be activated. Activation of classical complement pathway leads to the lysis of the cell through generation of membrane attack complex (MAC) (Fig. 12.1). Activation of complement cascade also leads to the formation of C3a and C5a fragments. These fragments are chemotactic for phagocytic cells. Fab portions of the antibodies bind to the cell bound antigens and complement gets fixed at the Fc portions of the antibody molecules. The phagocytes, which arrive at the site by the chemotactic activity of C3a and C5a possess complement receptors (CRs) and lead to the phagocytic response (Fig. 17.2).

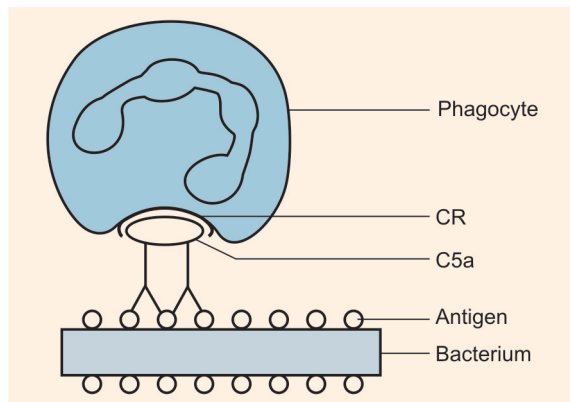


Fig. 17.2: Complement-mediated cytotoxicity.

2. Antibody-dependent cell-mediated cytotoxicity (ADCC)

This is the second possible mechanism involved in type II reactions. Fc portions of IgG or IgM adsorbed onto the target cell through Fab interact with Fc receptors (FcRs) of NK cell which, in this context of ADCC, is often referred to as a K cell. Macrophages and eosinophils also indulge in this type of activity. This leads to lysis of target cells (Fig. 17.3). Lysis of target cells requires contact with K cells but does not involve phagocytosis or fixation of complement.

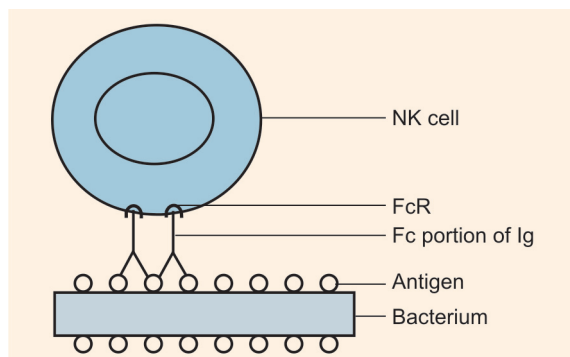


Fig. 17.3: Antibody-dependent cell-mediated cytotoxicity.

Clinically, antibody-mediated reactions occur in following situations.

1. Drug-induced immune haemolytic anaemia

A type II hypersensitivity reaction involving haemolytic anaemia may be induced by administration of certain drugs.

Drugs such as antibiotic penicillin, and alpha methyl dopa for the treatment of hypertension can bind to the surface of red blood cell and form an antigenic complex with the surface of these cells. This can bring about the production of complement-fixing antibody to the drug. Reaction of this antibody with the RBC-bound drug activates the complement system, resulting in RBC lysis and anaemia.

2. Transfusion reactions

When a person receives incompatible blood transfusion, the antibodies normally present in recipient's serum agglutinate and lyse donor RBCs with the liberation of free haemoglobin into the plasma. This leads to jaundice, fever and failure of kidney function. Serum of the donor may also agglutinate and lyse recipient's RBCs. For example, transfusion of group O whole blood or plasma to group A or group B or group AB recipients may lead to haemolysis. But this reaction is mild since the donor's serum gets considerably diluted in the circulation of the recipient.

3. Rh incompatibility

Rh antibodies are major cause of **haemolytic disease of newborn (HDN)**. When an Rh-negative woman carries an Rh-positive foetus, she may be immunized against Rh antigen by passage of foetal red cells into maternal circulation. Minor transplacental leaks may occur any time during pregnancy, but it is during delivery that foetal cells enter the maternal circulation in large numbers. Therefore, the mother is usually immunized only at first delivery, and consequently, the first child escapes damage (except where the woman has been sensitized already by prior Rh incompatible blood transfusion). During subsequent pregnancy, Rh antibodies of IgG class pass from the mother to the foetus and damage its erythrocytes. The clinical features of HDN may vary from a mere accentuation of the physiological jaundice in the newborn to erythroblastosis fetalis or intrauterine death due to **hydrops fetalis**.

4. Autoimmune haemolytic anaemia, agranulocytosis and thrombocytopenia

Some persons develop antibodies against their blood elements, resulting in autoimmune haemolytic anaemia, agranulocytosis or thrombocytopenia.

5. Anaemia due to infectious diseases

A variety of infectious diseases due to *Salmonella* and mycobacteria are associated with haemolytic anaemia. Studies in *Salmonella* infections have revealed that the haemolysis is due to an immune reaction against a lipopolysaccharide bacterial endotoxin that becomes coated onto the erythrocytes of the patient.

TYPE III HYPERSENSITIVITY: IMMUNE COMPLEX

If an antigen is not cell-bound but is rather small and soluble, the body can encounter severe difficulties if it is repeatedly exposed to that antigen. The formation of antigen-antibody complexes in serum, with subsequent deposition of the complexes in tissues, is the key event in type III hypersensitivity.

Antigen–antibody complexes can then activate serum complement and phagocytes, which lead to tissue damage.

Arthus reaction (local immune complex disease)

Arthus (1903) observed that when rabbits were repeatedly injected subcutaneously with normal horse serum, the initial injections were without any local effect, but with later injections, there occurred intense local reaction consisting of oedema, induration and haemorrhagic necrosis. This is known as Arthus reaction. The tissue damage is due to formation of local precipitating immune complexes which are deposited on the endothelial lining of the blood vessels. Antigen–antibody complexes can then trigger and activate complement leading to inflammation and tissue damage.

Serum sickness (systemic immune complex disease)

This is a systemic form of type III hypersensitivity. This develops in persons who receive a single injection of a high concentration of horse antitoxin against tetanus, gas gangrene, diphtheria, etc. for prophylactic and therapeutic purposes. Seven to 12 days after the injection, patient may develop fever, lymphadenopathy, splenomegaly, arthritis, glomerulonephritis, endocarditis, vasculitis, urticarial rash, abdominal pain, nausea and vomiting. It is due to the immune response to horse antigens and it has also been encountered in allergic reactions to certain drugs. After 7–12 days, antibodies to horse antigens appear while horse antigens are still persisting because a large dose was administered. Initially, they form antigen–antibody complexes in antigen excess. These soluble complexes can circulate and get deposited in various sites throughout the body particularly in skin, joints, kidneys and heart.

Antigen–antibody aggregates can fix complement leading to inflammation and tissue damage. The plasma level of complement falls due to massive complement activation and fixation by antigen–antibody complexes. Initially, the circulating immune complexes are in antigen excess and produce inflammatory lesions, but as antibody production rises, the immune complexes increase in size as zone of equivalence is reached. These larger immune complexes are more easily phagocytosed and cleared by the cells of reticuloendothelial system of liver and spleen. Once all

immune complexes are removed from the circulation, symptoms are usually resolved within a week.

TYPE IV HYPERSENSITIVITY: CELL-MEDIATED OR DELAYED

Type IV hypersensitivity or delayed hypersensitivity (DH) is the clinically observable outcome of cell-mediated immune reaction in the tissues of a sensitized individual. The reaction is not brought about by circulating antibodies and B lymphocytes but by sensitized T lymphocytes and macrophages. It is named delayed hypersensitivity because it appears in 24–48 hours after the presensitized host encounters the antigen, while immediate hypersensitivity reactions develop in 1/2 to 8 hours (Table 17.1). Two types of DH are recognized—tuberculin (infection) type and contact dermatitis type.

1. Tuberculin (infection) type

The immune response to the tubercle bacillus, observed by Robert Koch in 1880, has served a general model for DH. When a small dose (1 to 3 units) of tuberculin or purified protein derivative (PPD) is injected intradermally in an individual sensitized to tuberculo-protein by prior infection or immunization, an indurated inflammatory reaction, 10 mm or more in diameter, develops at the site of injection within 48–72 hours. It is characterized by erythema due to increased blood flow to the damaged area and induration due to infiltration with a large number of mononuclear cells, mainly T lymphocytes and about 10–20% macrophages. In unsensitized individuals, the tuberculin injection provokes no response. Cell-mediated hypersensitivity reactions are seen in a number of chronic infectious diseases due to mycobacteria, protozoa and fungi.

2. Contact dermatitis type

Contact dermatitis is cell-mediated allergic reaction that occurs when certain substances like metals (nickel and chromium), dyes (picryl chloride and dinitrochlorobenzene), drugs such as penicillin, and toiletries come in contact with skin. Sensitization is particularly liable to occur when contact is with an inflamed area and when chemical is applied in an oily base. Application of antibiotic ointments frequently provokes sensitization. The substances involved are not anti-

Table 17.1: Comparison of type I–IV hypersensitivity reactions

Characteristic	Type I	Type II	Type III	Type IV
Approximate time to develop clinical signs	1/2–8 hours	5–12 hours	3–8 hours	24–48 hours
Reaction mediators	IgE, histamine, serotonin, SRS-A, etc.	IgG, IgM and complement	IgG, IgM, complement, neutrophils, eosinophils and lysosomal enzymes	T cells, macrophages and lymphokines
Response to intradermal injection of antigen	Wheal and flare	—	Erythema and oedema	Erythema and induration
Passive transfer with	Serum	Serum	Serum	T cells
Examples	Anaphylaxis, asthma, hay fever, and food and insect allergies	Transfusion reaction, HDN and drug-induced allergy	Arthus reaction and serum sickness	Tuberculin test, contact dermatitis, graft rejection and tumour immunity

genic by themselves but may acquire antigenicity on combination with skin proteins. Subsequent contact with allergen in a sensitized individual leads to contact dermatitis. The lesions vary from macules and papules to vesicles which break down leaving behind raw weeping areas typical of acute eczematous dermatitis.

This hypersensitivity can be detected by **patch test**. The allergen is applied to the skin under an adherent dressing. Sensitivity is indicated by itching, appearing in 4–5 hours and local reaction which may vary from erythema to vesicle or blister formation in 24 hours.

TYPE V HYPERSENSITIVITY: STIMULATORY OR ANTIRECEPTOR

This is an antibody-mediated hypersensitivity. Here antibody reacts with a key surface component such as a hormone receptor and switches on or stimulates the cell. An example of this type of hypersensitivity is the thyroid hyperactivity in Graves' disease due to **thyroid-stimulating autoantibody**.

Normally, thyroid stimulating hormone (TSH) from pituitary gland binds to thyroid cell receptors. This activates adenylyl cyclase in the membrane which converts ATP to AMP. The latter stimulates activity of thyroid cells, thus secreting thyroxine. **The thyroid-stimulating antibody present in the sera of thyrotoxic patients is an autoantibody directed against receptors for TSH.** This antibody, therefore, binds to these receptors and brings about the same effect as that of TSH. Type V hypersensitivity differs from type II, because instead of binding to cell surface components, the antibodies, in type V hypersensitivity, recognize and bind to the cell surface receptors and stimulate the cell.

SHWARTZMAN REACTION

It is not an immune reaction. It is, however, traditionally described along with hypersensitivity reactions because of superficial resemblance. Schwartzman (1928) observed that when culture filtrate of Gram-negative bacteria is injected intradermally in rabbit and then after 24 hours a larger dose of the same or unrelated toxin is injected intravenously, within a few hours after second injection, the site of intradermal injection shows petechial haemorrhagic area. The absence of specificity and short interval between the two doses excludes the chances of immunological basis.

Mechanism

The first dose is called **preparatory dose** and the second dose is called **provocative dose**. The preparatory dose causes accumulation of leucocytes which release lysosomal enzymes. These enzymes damage capillary walls. Following provocative dose, there occurs intravascular clotting, the thrombi leading to necrosis of vessel walls and haemorrhage. This is called **local form of Schwartzman reaction**.

When both injections are given intravenously the animal dies 12–24 hours after the second dose. Autopsy reveals bilateral cortical necrosis of kidneys and patchy necrosis of the liver, spleen and other organs. This is known as **generalized Schwartzman reaction**. In fulminating meningococcal septicaemia (**Waterhouse-Friderichsen syndrome**), there occurs adrenal haemorrhage and profound shock. These manifestations may be due to Schwartzman-like phenomenon.

KEY POINTS

- **Type I hypersensitivity** is mediated by **IgE antibodies**.
- **IgE antibodies bind to specific receptors on mast cells and basophils.**
- **Type I hypersensitivity leads to anaphylaxis, hay fever, atopic dermatitis and allergic asthma.**
- Severity of symptoms depends on IgE antibodies and the quantity of allergen.
- **Type II hypersensitivity** is mediated by IgG or IgM antibodies against cell surface or extracellular matrix antigens.
- Antibodies damage cells and tissues by activating complement.
- **Haemolytic disease of newborn** occurs when maternal antibodies to foetal blood group antigen cross the placenta and destroy the foetal erythrocytes.
- **Persistence of antigen** from continued infection or in autoimmune disease can lead to **immune complex disease**.
- **Immune complexes** can form both in the circulation, leading to systemic disease, and at local sites such as the lungs.
- **Two types of type IV hypersensitivity** are recognized—tuberculin (infection) type and contact dermatitis type.

Important Questions

1. What is hypersensitivity? Classify hypersensitivity reactions. Describe type I reactions.
2. Describe type II hypersensitivity reactions. Briefly describe clinical examples.
3. What is the basis of type III hypersensitivity reactions? Discuss these giving suitable examples.
4. Discuss delayed hypersensitivity reactions.
5. Write short notes on:

(a) Prausnitz-Kustner reaction (c) Rh incompatibility (e) Serum sickness (g) Stimulatory hypersensitivity (i) Cutaneous anaphylaxis	(b) Desensitization (d) Arthus reaction (f) Contact dermatitis (h) Schwartzman reaction (j) Schultz-Dale reaction.
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Multiple Choice Questions

1. Anaphylactic reactions are mediated by which class of immunoglobulins?
 - (a) IgE.
 - (b) IgM.
 - (c) IgA.
 - (d) IgD.
2. Wheal and flare response is characteristic of:
 - (a) type I hypersensitivity reaction.
 - (b) type II hypersensitivity reaction.
 - (c) type III hypersensitivity reaction.
 - (d) type IV hypersensitivity reaction.
3. Raised level of immune complexes can be demonstrated in:
 - (a) poststreptococcal glomerulonephritis.
 - (b) lepromatous leprosy.
 - (c) hepatitis B.
 - (d) all of the above.
4. Delayed hypersensitivity reaction is mediated by:
 - (a) T lymphocytes.
 - (b) B lymphocytes.
 - (c) killer cells.
 - (d) natural killer cells.
5. Thyroid hyperactivity in Graves' disease is due to:
 - (a) type I hypersensitivity reaction.
 - (b) type II hypersensitivity reaction.
 - (c) type III hypersensitivity reaction.
 - (d) type V hypersensitivity reaction.
6. Drug-induced immune haemolytic anaemia is due to:
 - (a) type I hypersensitivity reaction.
 - (b) type II hypersensitivity reaction.
 - (c) type III hypersensitivity reaction.
 - (d) type IV hypersensitivity reaction.
7. Allergic rhinitis is mediated by which class of antibody?
 - (a) IgG.
 - (b) IgM.
 - (c) IgA.
 - (d) IgE.
8. Erythema and induration are characteristic of:
 - (a) type I hypersensitivity reaction.
 - (b) type III hypersensitivity reaction.
 - (c) type IV hypersensitivity reaction.
 - (d) type V hypersensitivity reaction.
9. Schultz-Dale phenomenon is a type of:
 - (a) type I hypersensitivity.
 - (b) type II hypersensitivity.
 - (c) type III hypersensitivity.
 - (d) type IV hypersensitivity.
10. Prausnitz-Küstner reaction is a type of:
 - (a) type I hypersensitivity.
 - (b) type II hypersensitivity.
 - (c) type III hypersensitivity.
 - (d) type IV hypersensitivity.
11. Which hypersensitivity reaction is T cell-mediated?
 - (a) Type I.
 - (b) Type II.
 - (c) Type III.
 - (d) Type IV.
12. A positive tuberculin test is an example of:
 - (a) type I hypersensitivity.
 - (b) type II hypersensitivity.
 - (c) type III hypersensitivity.
 - (d) type IV hypersensitivity.
13. An example of type III immune complex disease is:
 - (a) contact dermatitis.
 - (b) atopy.
 - (c) serum sickness.
 - (d) graft rejection.
14. Pollen would most likely evoke which type of hypersensitivity response?
 - (a) Type I.
 - (b) Type II.
 - (c) Type III.
 - (d) Type IV.
15. Which subclass of immunoglobulin G (IgG) can bind to mast cells?
 - (a) IgG1.
 - (b) IgG2.
 - (c) IgG3.
 - (d) IgG4.



Answers

- | | | | | | | | | | | | |
|-------|-------|-------|------|------|------|------|------|------|-------|-------|-------|
| 1. a | 2. a | 3. d | 4. a | 5. d | 6. b | 7. d | 8. c | 9. a | 10. a | 11. d | 12. d |
| 13. c | 14. a | 15. d | | | | | | | | | |

Autoimmunity*

Competency achievement: The student after reading the chapter should be able to:

MI1.10(b): Describe the immunological mechanisms of autoimmunity.

Normally, we do not form potentially destructive antibodies and T cells against our own cells because our body has developed tolerance to self-antigens. However, all mechanisms have a risk of breakdown. The self-recognition mechanisms are no exception, and a number of diseases have been identified in which there is autoimmunity, due to copious production of autoantibodies and autoreactive T cells. **Self-tolerance** refers to lack of responsiveness to an individual's own antigens, and obviously it underlines our ability to live in harmony with our own cells and tissues. **Autoimmunity may, therefore, be defined as immune response to self-antigens, which can generate autoantibodies and autoreactive T cells.**

MECHANISM OF AUTOIMMUNITY

There are several possible mechanisms involved in the development of autoimmunity:

1. Forbidden clones

According to clonal selection theory, antibody-forming lymphocytes capable of reacting with different antigens are formed. Clones of cells that have immunological reactivity with self-antigens are eliminated during embryonic life. Such clones are called forbidden clones. Their persistence or development in later life by somatic mutations can lead to autoimmunity.

2. Hidden or sequestered antigen

Certain self-antigens are present in closed systems and are not accessible to immune system. An example is the **lens antigen of the eye**. The lens protein is enclosed in its capsule and does not circulate in the blood. Therefore, immunological tolerance against this antigen does not develop during foetal

life. When the lens protein antigen leaks out, following cataract surgery or injury to the eye, it leads to immune response and damage to the other eye.

Another example of hidden antigen is seen in case of **sperm antigens**. Since sperms develop only at puberty, therefore, the sperm antigens cannot induce tolerance during foetal life. Sperms may enter into bloodstream following injury of the testes and mumps. Virus probably damages the basement membrane of seminiferous tubules leading to the leakage of sperms and initiation of an immune response resulting in orchitis.

3. Neoantigens or altered antigens

Cells or tissues may undergo antigenic alteration by physical agents such as irradiation. Photosensitivity or cold allergy may be due to altered antigens by light and cold, respectively. Several chemical agents including drugs can combine with cells and tissues and alter their antigenic structure. Skin contact with a variety of chemicals may lead to contact dermatitis. Drug-induced anaemia, leucopenia and thrombocytopenia often have autoimmune basis. Viruses and other intracellular pathogens may induce alterations of cell antigens leading to autoimmunity.

4. Cross reacting antigens

Immunological damage may result from immune response induced by cross reacting foreign antigens. For example, **Semple rabies vaccine** consists of infected sheep brain tissue inactivated with phenol. Its injection elicits an immune response against sheep brain antigens. This may lead to damage to patient's nervous tissue due to cross reaction between human and sheep brain leading to encephalitis.

Immunological injury may be due to cross reacting antigens present on microorganisms causing infection. An important example of this is the **non-suppurative sequelae of *Streptococcus pyogenes*** infection which include acute rheumatic fever and acute glomerulonephritis. M protein of *S. pyogenes* and heart of man share antigenic characteristics. The immune response induced by repeated streptococcal

* This chapter has been contributed by Dr. Paramjeet S. Gill, Professor, Department of Microbiology, PGIMS, Rohtak.

infections can, therefore, damage the heart. Nephritogenic strains of *S. pyogenes* share antigens with renal glomeruli. Therefore, immune response following infection with such strains may lead to acute glomerulonephritis.

Escherichia coli O14 shares antigen with human colon. This organism has, therefore, been blamed to cause ulcerative colitis. Many patients suffering from syphilis develop haemolytic anaemia. It has been suggested that antibodies raised against *Treponema pallidum* antigens cross react with certain blood group antigens bringing about the anaemia. There is also an evidence that antigens common to *Trypanosoma cruzi*, causative agent of Chagas' disease, and human cardiac muscle produce the immunopathological lesions seen in this disease.

5. Mutation

Immunocompetent cells may acquire an unnatural responsiveness to self-antigens by mutation.

6. Activity of helper and suppressor T cells

Helper T (Th) cells facilitate B cell response to many antigens. Suppressor T (Ts) cells inhibit antibody production by B cells. Optimal antibody response depends on the balanced activity of Th and Ts cells. *Overactivity of Th cells or decreased activity of Ts cells may lead to autoimmunity.*

CLASSIFICATION OF AUTOIMMUNE DISEASES

1. Autoimmune anaemias
 - (a) Pernicious anaemia
 - (b) Autoimmune haemolytic anaemias
2. Autoimmune thrombocytopenia
3. Autoimmune thyroid diseases
 - (a) Graves' disease (thyrotoxicosis)
 - (b) Hashimoto's disease (hypothyroidism)
4. Addison's disease
5. Autoimmune orchitis
6. Diabetes mellitus
7. Goodpasture's syndrome
8. Multiple sclerosis
9. Myasthenia gravis
10. Systemic autoimmune diseases
 - (a) Rheumatoid arthritis
 - (b) Systemic lupus erythematosus
 - (c) Systemic sclerosis (scleroderma)
 - (d) Dermatomyositis
 - (e) Polyarteritis nodosa
 - (f) Sjögren's syndrome

1. Autoimmune anaemias

(a) Pernicious anaemia

This is a type of megaloblastic anaemia caused by malabsorption of vitamin B₁₂ from the gastric mucosa. The causes of this malabsorption may be:

- Absence or decrease in intrinsic factor (IF) secreted by the gastric parietal cells.
- An autoimmune mechanism which leads to the production of antibodies either against the parietal cells which secrete IF, or against the IF itself.

Both these causes affect the absorption of vitamin B₁₂ into the bloodstream, leading to megaloblastic anaemia.

(b) Autoimmune haemolytic anaemias

These anaemias occur because of the production of antibodies against the patient's own red cells.

2. Autoimmune thrombocytopenia

In this disorder, platelets are destroyed by the formation of antiplatelet antibodies. Immunologically mediated destruction of platelets occurs in many different settings, including systemic lupus erythematosus, AIDS, following viral infections, and as a complication of drug therapy.

3. Autoimmune thyroid diseases

(a) Graves' disease (thyrotoxicosis)

Thyroid changes in Graves' disease are autoimmune in origin and initiated by thyroid stimulating antibody. The latter is an autoantibody directed against receptors for thyroid stimulating hormone (TSH). This antibody, therefore, binds to these receptors and brings about the same effect as that of TSH (*see* type V hypersensitivity, Chapter 17).

(b) Hashimoto's disease (hypothyroidism)

It is a disease of the thyroid which is most common in middle-aged women and often leads to formation of goitre and hypothyroidism. The gland is infiltrated with inflammatory lymphoid cells. The serum of the patient usually contains antibodies to thyroglobulin. Many patients also have antibodies to thyroid peroxidase, the enzyme which iodates thyroglobulin.

4. Addison's disease

Two important causes of Addison's disease are autoimmune adrenalitis and tuberculous adrenalitis. Autoimmune adrenalitis is suggested by lymphocytic infiltration of adrenal gland and the presence of circulating antiadrenal antibodies. Similar lesions can be produced in experimental animals by immunization with adrenal tissue in Freund's adjuvant.

5. Autoimmune orchitis

Twenty percent of males over the age of 13 years who are infected with mumps virus develop orchitis (often unilateral). Lymphocytic infiltration of the testis and circulating antibodies to the sperms and genital cells can be demonstrated in this condition.

6. Diabetes mellitus

Role of autoimmunity in the pathogenesis of diabetes is supported by following observations.

- Lymphocytic infiltrates, often intense, are frequently observed in the islets in cases of recent onset. Both CD4+ and CD8+ T cells are found within such infiltrates.
- As many as 90% of the patients with type I diabetes have circulating islet cell antibodies when tested within a year of diagnosis.

- Approximately 10% of persons who have type I diabetes also have other organ-specific autoimmune disorders, such as Graves' disease, Addison's disease, and pernicious anaemia.

7. Goodpasture's syndrome

Goodpasture's syndrome is characterized by the simultaneous appearance of proliferative, usually rapidly progressive glomerulonephritis and a necrotizing haemorrhagic interstitial pneumonitis. These lesions are the consequence of antibodies evoked by antigens present in the glomerular and pulmonary basement membrane.

8. Multiple sclerosis

There is a strong possibility that multiple sclerosis (MS) is the result of an immunological reaction. This is supported by following observations.

- The CSF and sera of MS patients contain many lymphocytes and show raised immunoglobulins.
- The early lesions often show lymphocytes (mostly CD4+ and CD8+ T cells) and plasma cells.
- Immunofluorescence studies have shown the presence of immunoglobulin in the lesional area.
- Serum from MS patient can induce myelinolysis in tissue culture.

9. Myasthenia gravis

Myasthenia gravis (MG) is considered to be an autoimmune disorder. Antibodies form against acetylcholine receptors (AChRs) present on the postsynaptic membrane of the neuromuscular junction. The antibodies interact with AChRs and cause their destruction through activation of complement system. Circulating antibodies to AChRs have been demonstrated in nearly all patients with myasthenia gravis, and the disease can be passively transferred to experimental animals by injecting serum from MG patient.

10. Systemic autoimmune diseases

(a) Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disorder that may affect many tissues and organs—skin, blood vessels, heart, lungs, and muscles, but principally attacks the joints producing a non-suppurative proliferative synovitis that often progresses to destruction of articular cartilage and ankylosis of joints. It is generally considered to be an autoimmune disease. Both humoral and cell-mediated immune reactions are believed to be important in the pathogenesis of RA.

The synovial membrane is usually infiltrated by large number of lymphocytes and plasma cells. There is an increased amount of immunoglobulins (IgG, IgM and IgA) in the serum and synovial fluid and there is deposition of immune complexes in the synovium. **About 80% of individuals with RA have autoantibodies to the Fc portion of autologous IgG called rheumatoid factors (RF).** These are mostly IgM antibodies but they may be of other classes (IgG and IgA). RF may be absent in some (seronegative)

and may be found in some non-RA patients such as systemic lupus erythematosus, dermatomyositis and even in otherwise healthy people. RF is detected by agglutination of latex particles coated with IgG.

(b) Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a chronic multisystem disease of autoimmune origin. Patients have a variety of antibodies directed against cell nuclei, intracytoplasmic cell constituents, immunoglobulins, thyroid and other organ specific antigens.

Antinuclear antibodies (ANAs) are directed against several nuclear antigens. Several techniques are used to detect ANAs. The most commonly used method is indirect immunofluorescence.

The immunofluorescence test for ANAs is positive in virtually every patient with SLE. Therefore, this test is very sensitive, but it is not specific because patients with other autoimmune diseases are frequently positive. Furthermore, 5–15% of normal individuals have low titres of these antibodies. Anti-DNA antibodies can also be detected by RIA or ELISA.

In tissues, nuclei of damaged cells react with ANAs, lose their chromatin pattern, and become homogenous, to produce so called **LE bodies**. Related to this phenomenon is the **LE cell**, which is readily seen *in vitro*. Basically the LE cell is any phagocytic leucocyte (neutrophil or macrophage) that has engulfed the denatured nucleus of an injured cell. For the demonstration of LE cells, the withdrawn blood is agitated with the beads in the tube for 30 minutes either manually or on a rotator. It is then incubated at 37°C for 30 minutes to 1 hour, shaking intermittently thus exposing their nuclei to ANAs. The binding of ANAs to nuclei denatures them, and subsequent fixation of complement renders antibody-coated nuclei strongly chemotactic for phagocytic cells. The blood is centrifuged, the cells from the buffy coat are withdrawn, thin-smear is prepared and stained with Leishman stain. It is examined under oil-immersion objective for **LE cells** which are indicated by bluish purple mass in the cytoplasm of a neutrophil. The LE cell test is positive in up to 70% of the patients with SLE.

Antinuclear antibodies can also be detected by agglutination of latex particles coated with deoxyribonucleoprotein. It is more specific and sensitive than LE cell test.

(c) Systemic sclerosis (scleroderma)

Systemic sclerosis is a chronic disease of unknown etiology characterized by abnormal accumulation of fibrous tissue in the skin and multiple organs. The skin is most commonly affected, but the gastrointestinal tract, kidneys, heart, and lungs also are frequently involved. In some patients, the disease appears to remain confined to the skin for many years, but in the majority, it progresses to visceral involvement with death from renal failure, cardiac failure, pulmonary insufficiency, or intestinal malabsorption.

Systemic sclerosis can be classified into two groups on the basis of its clinical course:

- **Diffuse scleroderma**, characterized by initial wide-spread skin involvement, with rapid progression and early visceral involvement.
- **Limited scleroderma**, with relatively minimal skin involvement, often confined to the fingers and face. Involvement of the viscera occurs late, and hence the disease in these patients generally has a fairly benign course.

(d) Dermatomyositis

Dermatomyositis is an inflammatory disorder of the skin as well as skeletal muscles. It is characterized by a distinctive skin rash that may accompany or precede the onset of muscle disease. The disease occurs most frequently during childhood and between the fourth and sixth decade of life. Most studies have found a preponderance of female patients over male patients.

It usually begins with a symmetric and painless weakness of the proximal muscles of the arms, legs, and trunk. The weakness is progressive and characteristically spreads to the face, neck, larynx, pharynx, and heart. Muscle involvement

may become severe enough to confine the patient to bed or cause death due to failure of the respiratory muscles.

(e) Polyarteritis nodosa

Polyarteritis nodosa (PAN) is a non-infective, acute necrotising vasculitis involving the small and medium arteries leading to weakness of vessel wall, aneurysm formation and thrombus formation in some cases. Though it has been suggested that PAN may be an autoimmune disease, the antibody responsible has not been identified. However, immune complexes of hepatitis B surface antigen in affected tissues, including the kidneys, have been demonstrated in 30–40% of patients.

(f) Sjögren's syndrome

Sjögren's syndrome is a clinicopathologic entity characterized by dry eyes (keratoconjunctivitis sicca) and dry mouth (xerostomia) resulting from immunologically mediated destruction of the lacrimal and salivary glands. This syndrome may occur in association with other autoimmune diseases, e.g. rheumatoid arthritis and SLE. Antinuclear antibodies and rheumatoid factor commonly occur in the patient serum.

KEY POINTS

- **Self tolerance** refers to *lack of responsiveness to an individual's own antigens*.
- **Autoimmunity** may be defined as *immune response to self antigens*.
- Virtually all autoimmune diseases studied have shown an *association with some HLA specificity*.
- **Autoimmunity** may be due to *forbidden clones, sequestered antigens, altered antigens, cross reacting antigens, mutation, and overactivity of Th cells or decreased activity of Ts cells*.



Important Question

What is autoimmunity? Describe various mechanisms involved in the development of autoimmunity giving suitable examples and classify various autoimmune diseases.



Multiple Choice Questions

- Overactivity of Th cells may lead to:
 - optimal antibody response.
 - optimal cell-mediated response.
 - autoimmunity.
 - none of the above.
- Pathogenesis of rheumatic fever is due to immunological injury caused by:
 - cross reacting antigens.
 - neoantigens.
 - forbidden clones.
 - hidden antigens.
- Lens antigens of the eye are:
 - sequestered antigens.
 - cross reacting antigens.
 - neoantigens.
 - none of the above.



Answers

1. c 2. a 3. a

Histocompatibility Systems

Competency achievement: The student after reading the chapter should be able to:

MI1.11: Describe the immunological mechanisms of transplantation and tumour immunity.

PA7.5: Describe the immunology and the immune response to cancer.

PA9.3: Describe the HLA system and the immune principles involved in transplant and mechanisms of transplant rejection.

SU13.1: Describe the immunological basis of organ transplantation.

Transplantation of normal tissues and organs from one animal to another has been extensively studied. Most of the experimental work on transplantation has been done on mice, an animal species easy to breed and handle in the laboratory. The aim of tissue and organ transplantation from one person to another is to replace diseased tissues and organs. The tissue or organ transplanted is known as the **transplant** or **graft**. The individual from whom the transplant is obtained is known as **donor** and the individual on whom it is applied, the **recipient**.

TYPES OF GRAFTS

1. **Autografts:** Grafts from one part of the body to another in the same individual are known as autografts. Autografts survive and function for the life time of the individual.
2. **Isografts:** Grafts between genetically identical individuals (identical twins).
3. **Allografts:** Grafts between members of the same species (allogeneic individuals) but of different genetic constitution.
4. **Xenografts:** Grafts between members of different species.

ALLOGRAFT REACTION

When a skin graft from an animal is applied on a genetically unrelated animal of the same species (allograft), for the first few days it behaves as an autograft. It is quickly vascularized

and looks healthy. By about fourth day, it slowly becomes dark because of diminished circulation due to stasis followed by thrombosis and haemorrhage. The graft is invaded by lymphocytes and macrophages and in about 2 weeks, the graft sloughs off due to ischaemic necrosis. In addition, antibodies are believed to play a significant role in this process. The sequence of events resulting in the rejection of an allograft is known as **first-set reaction**.

If in an animal, which has rejected a graft by first-set reaction, another graft from the same donor is applied, it will lead to a hyperacute or immediate rejection response. This is accomplished by cytolytic leucocytes and complement-mediated antibody lysis of transplanted cells. The initial vascularisation may not occur, if it does, it is poor and is halted abruptly. Thrombosis of vessels is a feature. The graft is rejected much sooner in 3 to 5 days. This is known as **second-set reaction**.

The cells of an individual express a unique set of membrane antigens called **histocompatibility antigens**, which immunologically define a person's cell type as specifically as do fingerprints. The information about the synthesis of these antigens is stored on **histocompatibility genes**. Everyone on earth, with the possible exception of identical twins, has a personal set of histocompatibility genes and gene products (histocompatibility antigens). These genetically defined histocompatibility antigens, some of which are found on the surface of all body cells, serve to discriminate self from non-self.

Only those grafts in which there is a complete identity between the genetic constitution of the donor and the recipient (their histocompatibility genes and antigens are identical) survive and function. The rejection of an allograft has an immunological basis. This is evident from the specificity of the second-set reaction. **Accelerated rejection is seen only if the second graft is from the same donor as the first.** Application of a skin graft from another donor will evoke only the first-set reaction.

MAJOR HISTOCOMPATIBILITY COMPLEX

Genes mediating graft rejection in mice are called the histocompatibility genes or H genes or H loci. A large number

of H loci exist widely spread throughout the mouse genome. The products of the different H genes differ greatly in their ability to induce graft rejection. The strongest locus is known as major histocompatibility complex (MHC) in contrast to weaker loci (minor histocompatibility loci) which are more than 30. The MHC in mouse is referred to as **H-2 complex**.

The MHC in humans is known as the **human leucocyte antigen (HLA) complex**. The genes that code for the HLA antigens are found on the short arm of sixth pair of chromosome (one paternal and one maternal). The MHC genes are contained within four HLA loci known as A, B, C and D (Fig. 19.1). There are many different alleles at each of HLA-A, HLA-B and HLA-C loci, although any individual will possess a maximum of two at each locus (one on each chromosome). The A locus is associated with segregant series of 24 alleles, the B locus with 52 alleles and the C locus with 11 alleles. The various alleles are assigned the letters signifying the locus followed by a number signifying the allele. For example, HLA-A10 implies an HLA allele number 10 belonging to the locus A.

There are three classes of genes in these HLA loci—class I, class II and class III genes which code for the corresponding molecules (antigens).

MHC class I molecules

Class I antigens are membrane-bound glycoprotein in nature. They are involved in recognition of target cells by cytotoxic T cells. The cytotoxic T cells recognize antigen only if presented simultaneously with class I antigens.

MHC class II molecules

They are glycoproteins consisting of two polypeptide chains (α and β) held together by non-covalent interactions. They are normally found on immunologically reactive cells such as B lymphocytes, macrophages, monocytes and activated T lymphocytes.

MHC class III molecules

The genes coding for the complement components of the classical (C2 and C4) and the alternative (properdin or factor B) pathway also reside in the MHC genes complex located between MHC class I and class II regions (Fig. 19.1).

HISTOCOMPATIBILITY TESTING

For matching of donor and recipient for transplantation following procedures are undertaken.

ABO grouping

When tissue transplantation is anticipated, grouping and crossmatching of blood from donor and recipient are performed as a first step. If there exists any discrepancy in the ABO blood group, then the use of the prospective donor's tissue is absolutely contraindicated because blood group antigens are strong histocompatibility antigens.

Tissue typing (detection of MHC antigens)

Class I antigens are identified by means of antisera, therefore, the term serologically defined antigens is applied to them. Antisera used to detect class I antigens are obtained from:

- multiparous women,
- individuals who have received multiple blood transfusions,
- individuals who have received and rejected grafts, and
- volunteers who have been immunized with cells from another individual with a different HLA haplotype.

Following methods are used.

(a) Lymphoagglutination test

When lymphocytes are mixed with a panel of specific antisera, agglutination of lymphocytes is seen with specific antiserum.

(b) Lymphocytotoxicity test

Lymphocytes are incubated with a panel of antisera directed against specific class I MHC antigens followed by addition of complement. Lysis of cells is seen with specific antiserum. This can be detected by the addition of eosin or trypan blue which stains only dead cells.

(c) Mixed lymphocyte culture assay

MHC class II antigens are identified by the method known as mixed lymphocyte culture (MLC) assay. This can determine a possible match between donor and recipient class II antigens. In this test, donor and recipient lymphocytes are mixed together in a tube containing a radioactive DNA precursor. Donor or stimulator cells are irradiated to prevent DNA synthesis and proliferation. If the class II antigens are foreign, the responder cells will be stimulated to divide. As the stimulated cells replicate their DNA, they incorporate the radioactive precursor. The amount of radioactivity incorporated into cells can then be easily measured and quantitated.

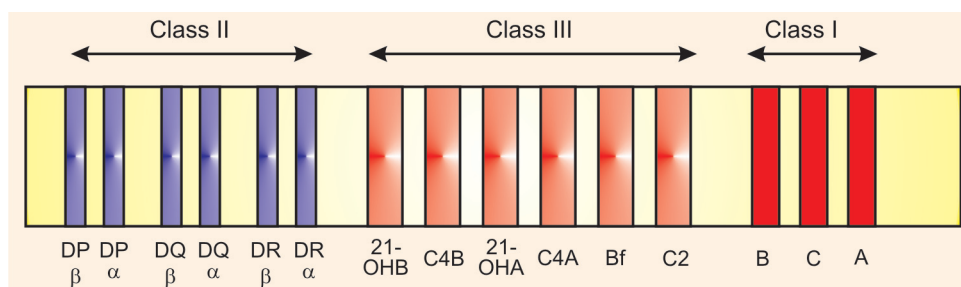


Fig. 19.1: HLA complex.

GRAFT VERSUS HOST REACTION

Under certain circumstances the immunologically competent cells of the graft react against antigens of the host (recipient), the reverse of the normal transplantation reaction. Such a reaction is known as graft versus host (GVH) reaction. Following conditions are necessary for the development of GVH reaction.

1. The host's immunological responsiveness must be either destroyed or so impaired (following whole-body irradiation) that he cannot reject a graft (allograft or xenograft).
2. There is HLA incompatibility between the donor and the host (recipient).
3. The graft (bone marrow, lymphoid tissue, splenic tissue, etc.) contains immunocompetent cells. MHC antigens of recipient activate transplanted immunocompetent cells which lead to the production of antibodies, lymphokines, activated phagocytes, Tc cells, etc. They attack the recipient cells leading to the death of the recipient.

FOETUS AS A GRAFT

Foetus is always a mixture of maternal and paternal genes, therefore, foetal MHC antigens are always different from those of the mother. In spite of this, foetus is not treated as foreign transplanted tissue and rejected. On the other hand, it grows and develops inside the uterus of the mother for more than 9 months because of immunosuppressant factors made by foetus, placenta and mother.

1. Cells of the placenta produce mucoproteins which coat foetal cells, thus masking histocompatibility antigens and prevent recognition.
2. Placental giant cells can produce soluble inhibitory factor which suppresses T cell proliferation and antibody production, and induces a population of Ts cells. Immunosuppression is also due to hormones (human chorionic gonadotropin, produced by placenta, and the high levels of maternal progesterone produced during pregnancy).
3. The mother produces specific blocking antibodies to foetal antigens of the foetal cells thus blocking immune recognition and immune attack by maternal Tc cells.
4. Major foetal serum protein, α -fetoprotein, can stimulate the formation of Ts cells, and the amniotic fluid contains immunosuppressive phospholipids. These factors contribute to immunosuppression in the mother.
5. β_1 -glycoprotein of foetal origin can be found in maternal plasma. It has been shown to inhibit maternal cellular immunity.

These factors depress immune system of the mother, however, it is usually not life-threatening because most of the response is localized in the uterus.

Why does the foetus not reject the mother? It may be due to the fact that the developing immune system of the foetus cannot respond.

TUMOUR IMMUNOLOGY

When a cell undergoes malignant transformation, it acquires new surface antigens and may lose some normal antigens. A tumour can, therefore, be considered as an allograft and be expected to induce an immune response.

Tumour antigens

Tumour antigens that elicit an immune response have been demonstrated in many experimentally-induced tumours and in some human cancers. They can be broadly classified into two categories:

1. Tumour-specific antigens (TSAs)

They are present only on the membranes of malignant cells and not on normal cells. They induce immune response when tumour is transplanted to syngeneic animals. Different tumours possess different TSAs, even though induced by the same carcinogen. In contrast, TSA of virus-induced tumours is virus-specific in that all tumours produced by one virus possess the same antigen, even if the tumours are in different animal strains or species.

2. Tumour-associated antigens (TAAs)

These are present on tumour cells and also on some normal cells. Since they are also present on some normal cells, therefore, they do not evoke an immune response and are of little significance in tumour rejection. Detection of these antigens is nevertheless of value in the diagnosis of certain tumours and antibodies raised against them can be useful for immunotherapy. TAAs fall into three categories:

(i) Tumour-associated carbohydrate antigens (TACAs)

They represent abnormal forms of widely expressed glycoproteins and glycolipids such as mucin-associated antigen detected in pancreatic and breast cancers.

(ii) Oncofoetal antigens

These are foetal antigens which are found in embryonic and malignant cells, but not in normal adult cells. The best known examples are α -fetoprotein in hepatomas and carcino-embryonic antigen in carcinoma of the colon, pancreas, lung, stomach and breast.

(iii) Differentiation antigens

CD10, an antigen expressed in early B lymphocytes, is also present in B cell leukaemias and lymphomas. Similarly, prostate-specific antigen is expressed on normal as well as cancerous prostatic epithelium. Both serve as useful differentiation markers in the diagnosis of lymphoid and prostatic cancer.

Immune response in malignancy

Both cell-mediated and antibody-mediated immunity have antitumour activity. As with virus-infected cells and foreign grafts, the T lymphocytes play a major role in the destruction of tumour cells in mammals. T cell activation generates

helper T (Th), delayed-type hypersensitivity T (Td) and cytotoxic T (Tc) cells. Of special interest is the role played by Td cells. These cells affect tumour killing by means of the lymphokines that they release.

NK cells are lymphocytes that are capable of destroying tumour cells without prior sensitization. After activation with IL-2, NK cells can lyse a wide range of human tumours, including many that appear to be non-immunogenic for T cells. So NK cells may provide the first line of defence against many tumours. In addition to direct lysis of tumour cells, NK cells can also participate in antibody-dependent cellular cytotoxicity (ADCC).

Activated macrophages also exhibit some selective cytotoxicity against tumour cells *in vitro*. T cell derived cytokine (interferon-gamma) activates macrophages which acquire antitumour activity.

Humoral mechanisms may also participate in tumour cell destruction by activation of complement and induction of ADCC by NK cells.

Immunosurveillance

It has been postulated that the primary function of cell-mediated immunity is to destroy malignant cells that arise by somatic mutation. Such malignant mutations are believed to occur frequently and would develop into tumour but for the constant vigilance of the immune system. The strongest argument for the existence of immunosurveillance is the increased frequency of cancers in immunodeficient hosts. About 5% of persons with congenital immunodeficiencies develop cancers about 200 times the expected prevalence. Immunosuppressed transplant recipients and patients with AIDS have more malignancies. **Therefore, inefficiency of immunosurveillance, either as a result of ageing or in congenital or acquired immunodeficiencies, leads to an increased incidence of cancer.**

Tumour escape mechanism

Most cancers occur in persons who do not suffer from any overt immunodeficiency. It is, therefore, evident that tumour cells must develop mechanisms to escape or evade the immune system in immunocompetent hosts. Several such mechanisms may be operative:

Weak immunogenicity

Some tumours are weakly immunogenic, so in small numbers they do not elicit an immune response. But when their numbers increase enough to provoke immune response the tumour load may be too great for the host's immune system to mount an effective response.

Modulation of surface antigens

Certain tumour cells can transfer antigens from their surface to the cytoplasm or they may shed or stop expressing the surface antigens thus making the tumour cells immunologically invisible.

Masking tumour antigens

Certain cancers produce copious amounts of a mucoprotein called sialomucin. It binds to the surface of the tumour cells. Since sialomucin is a normal component, the immune system does not recognize these tumour cells as foreign.

Induction of immune tolerance

Some tumour cells can synthesize various immunosuppressants. They may also activate specific Ts cells. Both these suppress the effector T and B cell clones.

Production of blocking antibodies

Some tumour cells invoke immune system to produce blocking antibodies that cannot fix and activate complement, so lysis of tumour cell is not possible. Blocking antibodies also cover the surface of cancer cells, preventing Tc cells from binding to hidden receptors.

Reduced levels of HLA class I molecules

In some instances, tumour cells express reduced levels of HLA class I molecules. This impairs presentation of antigenic peptides to cytotoxic T cells.

Immunotherapy of cancer

Immunotherapy of cancer is of two types:

1. Antigen-non-specific treatment

This activates the cells of the immune system in a generalized manner which destroy the tumour cells. **BCG vaccine** when injected directly into certain solid tumours may lead to tumour regression. This may also be administered by scarification in which deep scratches are made in the thigh and BCG vaccine applied on the scratches. Antitumour effect of BCG is believed to be due to activation of macrophages and NK cells. **Treatment with BCG has been reported to be useful in malignant melanomas, stage I lung cancer, bladder cancer and certain leukaemias.**

Corynebacterium parvum has also been shown to possess antitumour activity. It appears to activate macrophages and B cell function. When used in conjunction with cyclophosphamide (chemotherapeutic agent) a synergistic antitumour effect is achieved. This therapy has been reported to be beneficial in various types of lung cancers and metastatic breast cancer.

Instead of using live bacteria, **synthetic immunostimulants** structurally derived from the bacterial cell walls of *Mycobacterium bovis* (muramyl dipeptides) and *C. parvum* (trehalose diesters) may be used. When used in combination, they act synergistically to give enhanced antitumour activity by activating macrophages against cancer cell.

Other **non-specific immune modulators** include thymic hormones (thymosine, thymopoietin, thymic humoral factor and thymic serum factor) to restore T cell function, interferon to stimulate NK cell function, tuftsin (tetrapeptide located in the H chains of γ globulin) to stimulate phagocytic cells

and IL-2 to stimulate killing of cancer cells by Tc cells, NK cells and macrophages. Bone marrow transplantation is another approach to the treatment of some neoplastic diseases such as leukaemia.

2. Antigen-specific treatment

This includes:

Vaccination with tumour antigens

This provides immunity against specific tumour type.

Treatment with immune RNA

This stimulates humoral and cell-mediated immune responses.

Treatment with transfer factor

This stimulates cell-mediated immune response and release of lymphokines.

Modification of tumour antigenicity

The immunogenicity of the tumour cell can be increased by treatment with neuraminidase, which removes sialomucin covering tumour antigens.

Monoclonal antibodies

These can be raised against TAAs. These, when administered, either alone or tagged with a cytotoxic drug, will bind to and specifically destroy only cancer cells.

KEY POINTS

- Cells of an individual express a unique set of membrane antigens called **histocompatibility antigens**.
- Major histocompatibility complex in humans is known as **human leucocyte antigen (HLA) complex**.
- **Genes** that code for HLA antigens are found on **the short arm of sixth pair of chromosome**.
- There are three **classes of genes** in HLA loci—**class I, class II and class III genes** which code for the corresponding molecules (antigens).
- **Immunologically competent cells** of the graft may react against **antigens of the host** (graft versus host reaction).
- When a cell undergoes malignant transformation, it acquires new surface antigens and may lose some normal antigens.



Important Questions

1. Define various types of grafts? Describe allograft reaction.
2. What is the mechanism of graft rejection?
3. What is major histocompatibility complex? Describe various classes of MHC molecules.
4. What are histocompatibility antigens? Describe various procedures for histocompatibility testing.
5. Name and describe various tumour antigens.
6. Discuss immunotherapy of cancer.
7. Write short notes on:
 - (a) Tumour escape mechanism
 - (b) Graft versus host reaction
 - (c) Foetus as a graft
 - (d) MHC restriction.



Multiple Choice Questions

1. Grafts between members of the same species but of different genetic constitution are known as:
 - (a) autografts.
 - (b) isografts.
 - (c) allografts.
 - (d) xenografts.
2. Application of a skin allograft for the second time from the same donor will result in:
 - (a) first-set reaction.
 - (b) second-set reaction.
 - (c) both of the above.
 - (d) none of the above.
3. Major histocompatibility complex in man is known as:
 - (a) H-2 complex.
 - (b) HLA complex.
 - (c) both of the above.
 - (d) none of the above.
4. Class I histocompatibility antigens are products of:
 - (a) HLA-A locus.
 - (b) HLA-B locus.
 - (c) HLA-C locus.
 - (d) all of the above.
5. Class II histocompatibility antigens are encoded by:
 - (a) HLA-DP locus.
 - (b) HLA-DQ locus.
 - (c) HLA-DR locus.
 - (d) all of the above.
6. Which of the following disease/s is/are associated with particular HLA antigens?
 - (a) Graves' disease.
 - (b) ankylosing spondylitis.
 - (c) rheumatoid arthritis.
 - (d) all of the above.

7. The first line of defence against many tumours is:

- (a) natural killer cells.
- (b) polymorphs.
- (c) monocytes.
- (d) None of the above.

8. Example/s of immunomodulator/s is/are:

- (a) *Mycobacterium bovis* cell wall.
- (b) *Corynebacterium parvum* cell wall.

(c) interferon.

(d) all of the above.

9. A transplant between individuals of different animal species is known as:

- (a) xenograft.
- (b) isograft.
- (c) homograft.
- (d) allograft.



Answers

1. c

2. b

3. b

4. d

5. d

6. d

7. a

8. d

9. a

Immunohaematology

Competency achievement: The student after reading the chapter should be able to:

PA22.5: Enumerate and describe infections transmitted by blood transfusion.

The ABO system is the most important of all the blood group systems and its discovery by Landsteiner (1900) made blood transfusion possible. Subsequently, other blood groups (MN, P, Rh, Lutheran, Lewis, Kell, Duffy, Kidd, Diego, Yt, Kg, Dombroc and Colton) were reported. ABO and Rh are the major blood group antigens.

ABO blood group system

The ABO system contains four blood groups. The blood group is determined by the presence or absence of two distinct antigens A and B on the surface of the erythrocytes. Distribution of ABO antigens on red blood cells and antibodies in the serum is shown in Table 20.1. An individual with blood group A possesses antigen A on its RBCs and its serum possesses anti-B antibodies. Similarly, B, AB and O groups possess B, A and B, and no antigen on their RBCs and anti-A, none, and anti-A and anti-B antibodies in their sera, respectively. Forty per cent individuals in India have O group followed by B (33%), A (22%) and AB (5%).

Table 20.1: Distribution of ABO antigens on the red blood cells and antibodies in the serum

Blood group	Antigens on red blood cells	Antibodies in serum	Occurrence (%) in India
A	A	Anti-B	22
B	B	Anti-A	33
AB	A and B	None	5
O	None	Anti-A and anti-B	40

Anti-A and anti-B isoantibodies appear in the serum of infants by about the age of 6 months and persist thereafter. These are called natural antibodies, because they are seen to arise without any apparent antigenic stimulation. However, it is believed that they develop as a result of cross-immunization with bacteria of family Enterobacteriaceae that

colonize the infant's gut. These bacteria have outer membrane oligosaccharides strikingly similar to those that define the A and B antigens in the human body. Therefore, a newborn with group A will not have anti-B in his or her serum, since there has been no opportunity to undergo cross-immunization. When the intestine is eventually colonized by the normal microbial flora, the infant will start to develop anti-B, but will not produce anti-A because of tolerance to his or her own blood group antigens.

Immune isoantibodies may develop following ABO incompatible pregnancy or transfusion. More commonly, they result from injection of substances containing blood group-like antigens such as horse serum or bacterial vaccines made from media containing horse serum. **Natural antibodies are saline agglutinating while immune isoantibodies are albumin agglutinating.** The latter generally cause more severe transfusion reactions.

Ideally, the donor and recipient should belong to the same ABO group. It used to be held that O group cells could be transfused to recipients of any group as they possessed neither A nor B antigen. Hence O group was designated as the '**universal donor**'. The anti-A and anti-B antibodies in the transfused O blood do not ordinarily cause any damage to red cells of A, B and AB group recipients because they will be rendered ineffective by dilution in the recipient's plasma. But some O group plasma may contain isoantibodies in high titres (1:200 or above) so that the damage to the recipient's cells may result. This is known as **dangerous O group**. Transfusion of large quantities of O group blood to persons of another group may also cause reactions. **Anti-A antibody in O group blood is generally more potent than anti-B.** Hence, O group is more likely to cause reaction when given to group A recipient, than to those of B group. *While O group blood may be transfused to a patient of any other group in dire emergency, this practice should never be employed as a routine.*

Due to the absence of isoantibodies in plasma, AB group persons were designated '**universal recipients**'. AB group donors may not always be available due to their rarity and it may, on occasion, be necessary to use donors of other groups.

In such cases **group A blood is safer than B, because anti-A antibody is usually more potent than anti-B.**

Blood groups are inherited by simple Mendelian laws. Their synthesis is determined by allelomorphic genes *A*, *B* and *O*. Genes *A* and *B* give rise to corresponding antigens but *O* does not produce any antigen.

H antigen

Red cells of all ABO groups possess a common antigen, the H antigen, which is the precursor for the formation of A and B antigens. Due to its universal distribution, H antigen is not ordinarily important in grouping or blood transfusion. However, Bhende et al. (1952) from Bombay (now Mumbai) reported a very rare instance in which A and B antigens as well as H antigens were absent from the red cells. This is known as **Bombay** or **Oh blood group**. Sera of these individuals have anti-A, anti-B and anti-H antibodies, therefore, they can accept the blood only from the same rare blood group.

A, B and H antigens are glycoproteins. In addition to erythrocytes, they are also present in almost all the tissues and fluids of the body. A and B antigens have also been extracted and purified commercially from stomach of hogs and horses. Anti-A antibodies can be obtained from the albumin gland of the snails. Because anti-A harvested from snails has a very high titre, as much as 2.5 litres of potent reagent can be prepared from one snail.

Rh blood group system

Rh antibodies are clinically most significant after anti-A and anti-B antibodies. Almost all Rh antibodies result from immunization by pregnancy or blood transfusion. Rh antibodies are major cause of **haemolytic disease of newborn (HDN)** and lead to destruction of transfused cells. When an Rh-negative woman carries an Rh-positive foetus, she may be immunized against Rh antigen by the passage of foetal red cells (Rh-positive) into maternal circulation. Minor transplacental leaks may occur any time during pregnancy, but it is during delivery that foetal cells enter the maternal circulation in large numbers. Therefore, the mother is usually immunized only at first delivery and consequently the first child escapes damage (except when the woman has already been sensitized by prior Rh incompatible blood transfusion). During subsequent pregnancy, Rh antibodies of the IgG class pass, through the placenta, from the mother to the foetus and damage its erythrocytes. The clinical features of HDN may vary from a **mere accentuation of the physiological jaundice in the newborn to erythroblastosis fetalis or intrauterine death due to hydrops fetalis.**

Most Rh antibodies belong to IgG class. Being incomplete antibodies, they do not agglutinate Rh-positive cells in saline. A minority are complete (saline agglutinating) antibodies of IgM class. These are not relevant in the pathogenesis of haemolytic disease as they do not cross the placenta.

HDN does not affect all issues of Rh-incompatible marriages. Its incidence is much less than the expected figures. This is due to:

1. **Immunological unresponsiveness to Rh antigens:** Not all Rh-negative individuals form Rh antibodies following antigenic stimulation. Some fail to do even after repeated injections of Rh-positive cells. They are called '**non-responders**'. The reason for this immunological unresponsiveness is not known.
2. **Foetomaternal ABO incompatibility:** Rh immunization is more likely to result when mother and foetus possess the same ABO group. When Rh and ABO incompatibility co-exists, Rh sensitization from the mother is rare. In this situation the foetal cells entering the maternal circulation are destroyed rapidly by the ABO antibodies before they can induce Rh antibodies.
3. **Number of pregnancies:** As explained above the first child usually escapes disease. The risk increases with each successive pregnancy.

Rh isoimmunization can be prevented by administration of 100–300 µg of anti-Rh IgG prepared from human volunteers, at the time when the antigenic stimulation is expected to take place, i.e. immediately after delivery. To be effective, this should be employed from first delivery onwards.

Complications of blood transfusion

The complications of blood transfusion may be divided into immunological and non-immunological.

Immunological complications

Immunological complications may be caused by red cell, leucocyte or platelet incompatibility or allergic reaction to plasma components.

Red blood cell incompatibility leads to acute intravascular haemolysis or the red blood cells may be coated by antibodies and engulfed by phagocytes, removed from the circulation and subjected to extravascular lysis. Haemolysis may also be due to transfusion of group O whole blood or plasma to group A or group B or group AB recipients.

Leucocyte incompatibility may cause fever, pulmonary infiltrates, dyspnoea, non-productive cough and chest pain. The risk of these reactions can be reduced by using leucocyte-poor red blood cells. Leucoagglutinins are probably the commonest cause of fever. Platelet incompatibility, allergy and infection may also lead to fever.

Allergic reactions may be caused by interaction of patient's preformed reagins with transfused allergens. When allergic reactions develop administer antihistaminics during transfusion.

Non-immunological complications

Non-immunological complications of blood transfusion include transmission of infectious agents and circulatory overload. Infectious agents which may be transmitted during blood transfusion may be viruses, bacteria and protozoa (Table 20.2). Circulatory overload may be due to massive transfusion.

Table 20.2: Non-immunological complications of blood transfusion

- I. Transmission of infectious agents
- Viruses
 - Hepatitis B virus*
 - Hepatitis C virus*
 - Human immunodeficiency virus 1 and 2*
 - Human T cell lymphotropic virus 1 and 2
 - Cytomegalovirus
 - Bacteria
 - *Treponema pallidum**
 - *Leptospira interrogans*
 - *Borrelia burgdorferi*
 - Protozoa
 - *Plasmodium* spp.*
 - *Babesia* spp.
 - *Trypanosoma cruzi*
 - *Leishmania donovani*
 - *Toxoplasma gondii*
- II. Circulatory overload

* Mandatory tests in India.

KEY POINTS

- **ABO blood group** is determined by the presence or absence of **two distinct antigens A and B** on the surface of erythrocytes.
- Forty percent individuals in India have O group followed by B (33%), A (22%) and AB (5%).
- Red blood cells of all ABO groups possess a common antigen, **the H antigen**, which is precursor for the formation of A and B antigens.
- Rarely, H antigen is absent from red cells; this is known as **Oh or Bombay blood group**.
- **Rh antibodies** are major cause of **haemolytic diseases of newborn** and lead to **destruction of transfused cells**.
- Most Rh antibodies belong to IgG class.
- **Maternofoetal ABO incompatibility** is very common but **only in a proportion of these haemolytic disease occurs** because in persons of blood group **A or B**, **natural antibodies are IgM in nature**, therefore, they cannot cross the placenta to harm the foetus.

Important Questions

1. Discuss ABO blood group system.
2. Name various blood group systems and discuss Rh blood group system.
3. What is haemolytic disease of newborn? Why is its incidence much less than the expected figures?
4. What are the complications of blood transfusion?

Multiple Choice Questions

1. An individual with blood group A possesses:
 - (a) antigen A on RBCs.
 - (b) anti-A antibodies in serum.
 - (c) none of the above.
 - (d) both of the above.
2. An individual with blood group B possesses:
 - (a) antigen B on RBCs.
 - (b) anti-B antibodies in serum.
 - (c) both of the above.
 - (d) none of the above.
3. An individual with blood group AB possesses:
 - (a) A and B antigens on RBCs.
 - (b) neither anti-A nor anti-B antibodies in serum.
 - (c) both of the above.
 - (d) none of the above.
4. An individual with blood group O possesses:
 - (a) neither A nor B antigens on RBCs.
 - (b) both anti-A and anti-B antibodies in serum.
 - (c) both of the above.
 - (d) none of the above.
5. Commonest ABO blood group in Indian individuals is:
 - (a) O.
 - (b) A.
 - (c) B.
 - (d) AB.

6. Least common ABO blood group in Indian individuals is:
(a) O. (b) A.
(c) B. (d) AB.
7. Haemolytic disease of newborn may occur when:
(a) an Rh-negative woman carries an Rh-positive foetus.
(b) an Rh-positive woman carrier an Rh-negative foetus.
(c) both of the above.
(d) none of the above.
8. Which of the following infectious agents can be transmitted by blood transfusion?
(a) Hepatitis B virus.
(b) Human immunodeficiency virus.
(c) *Plasmodium* spp.
(d) All of the above.
9. Immunological complications of blood transfusion may be due to:
(a) red cell incompatibility.
(b) leucocyte incompatibility.
(c) platelet incompatibility.
(d) all of the above.
10. Individuals with Bombay blood group lack:
(a) A antigen.
(b) B antigen.
(c) H antigen.
(d) all of the above.



Answers

1. a 2. a 3. c 4. c 5. a 6. d 7. a 8. d 9. d 10. d